Basic Instrumentation

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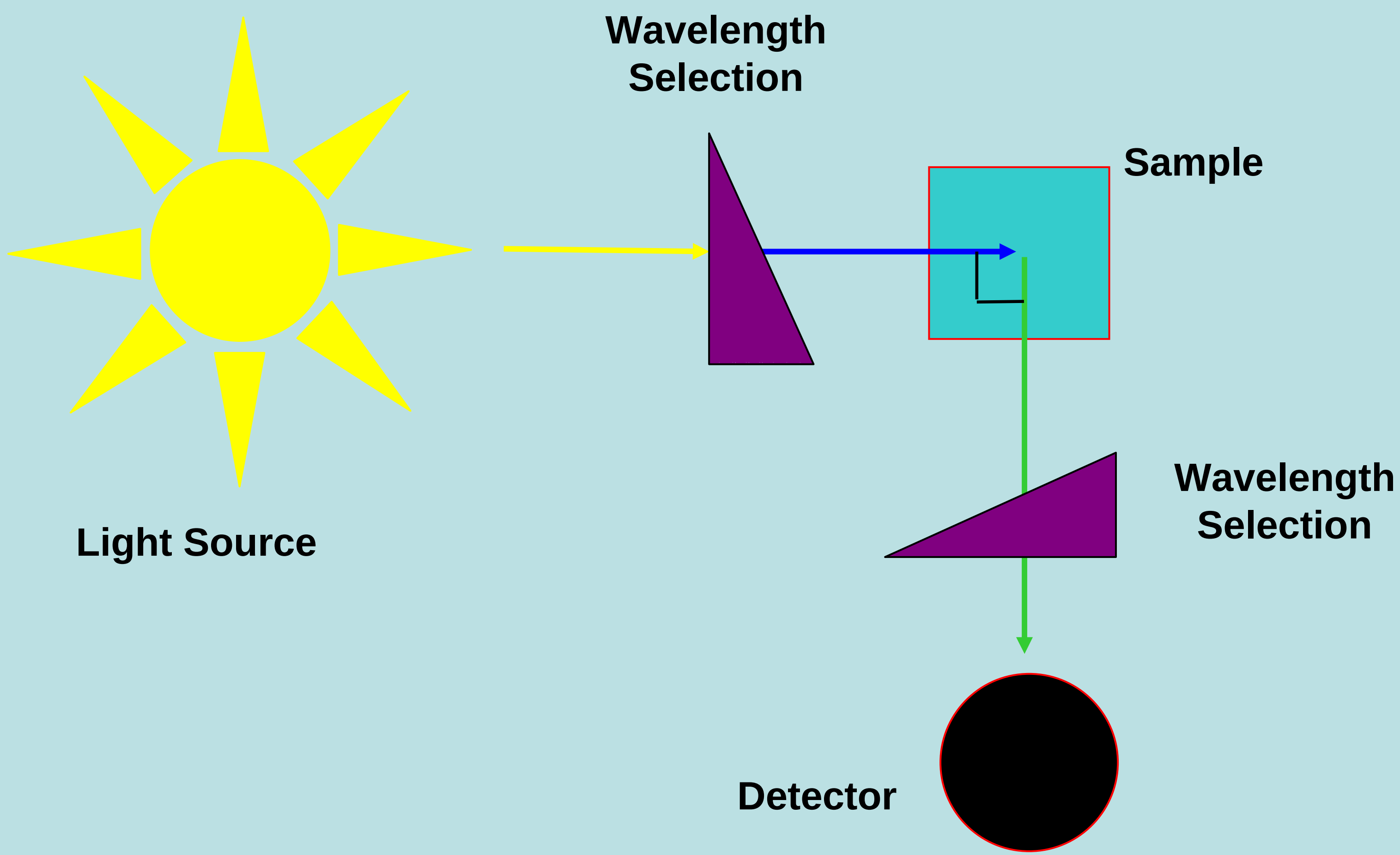
Principles of Fluorescence Techniques
Genova, Italy
June 14-16, 2004

ISS PC1 (ISS Inc., Champaign, IL, USA)

Fluorolog-3 (Jobin Yvon Inc, Edison, NJ, USA)

QuantaMaster (OBB Sales, London, Ontario N6E 2S8)

The Basics


Wavelength
Selection
Sample
Light Source
Wavelength
Selection
Detector

Fluorometer Components

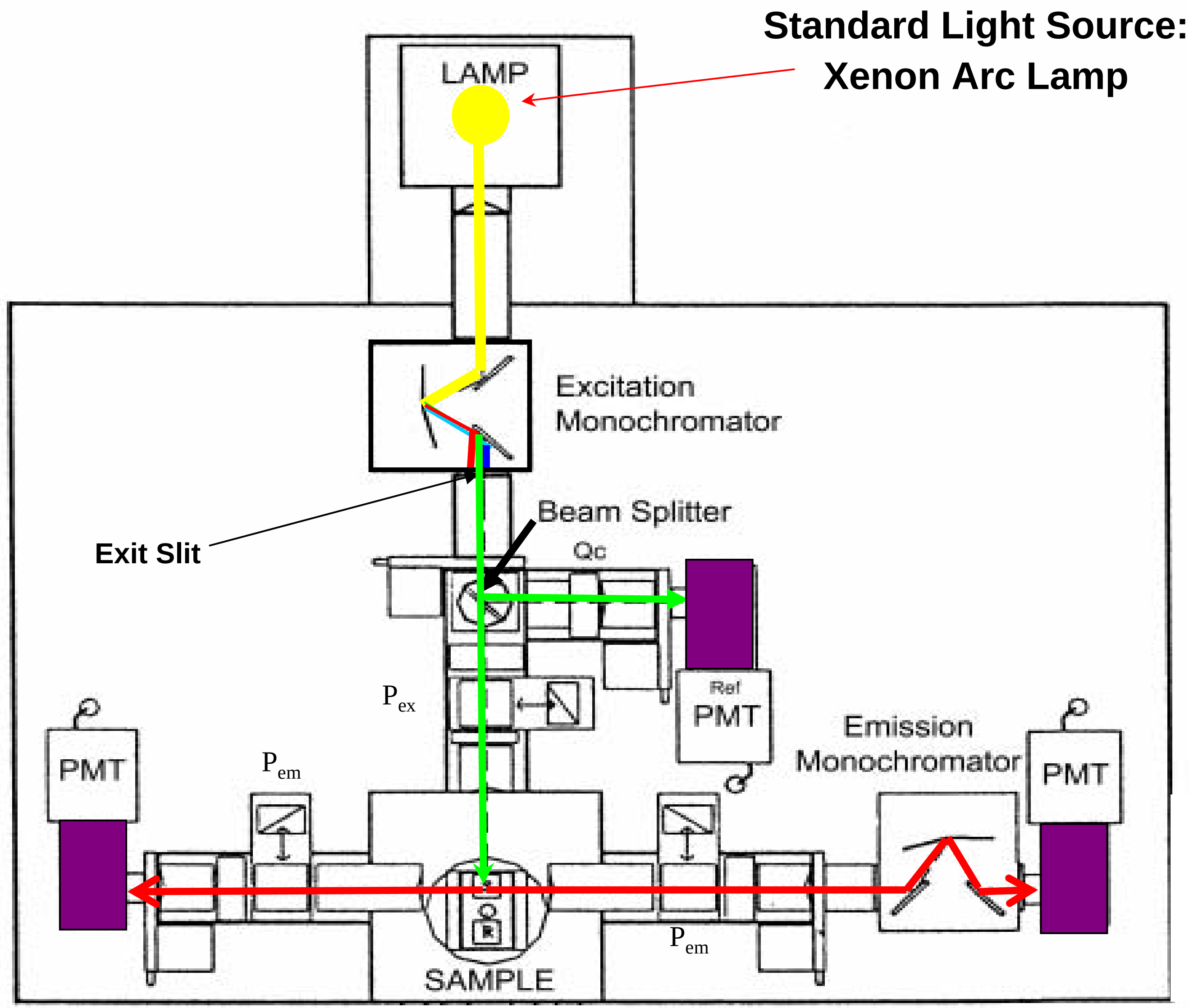
Light Source

Detectors

Wavelength Selection

Polarizers

The Laboratory Fluorometer



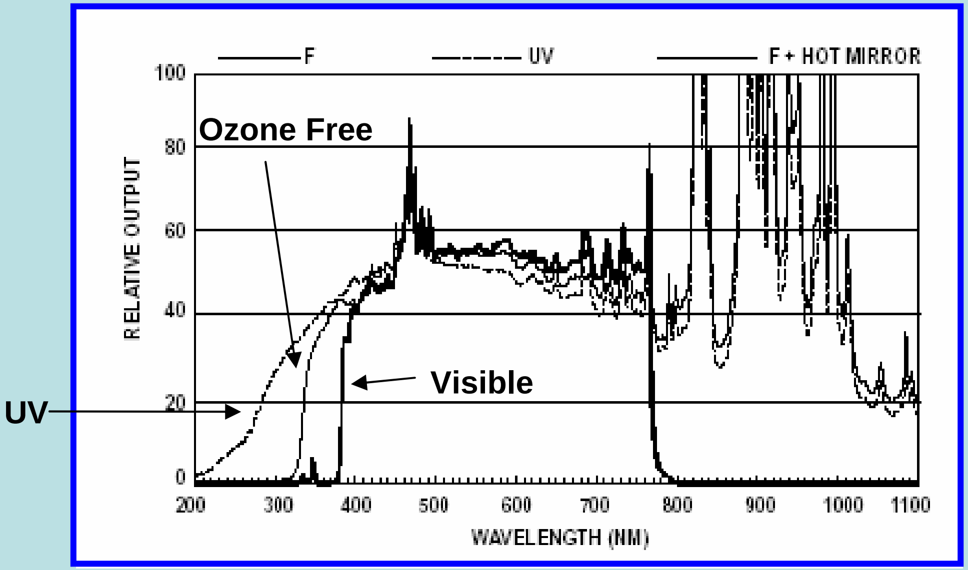
ISS (Champaign, IL, USA) PC1 Fluorometer

Light Sources

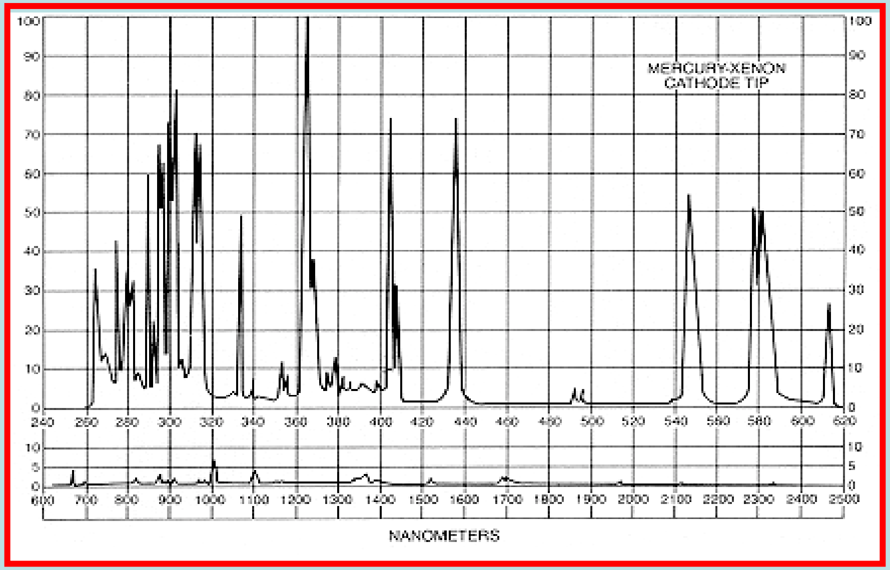
Lamp Light Sources

1. Xenon Arc Lamp (wide range of wavelengths)
2. High Pressure Mercury Lamps (High Intensities but concentrated in specific lines)
3. Mercury-Xenon Arc Lamp (greater intensities in the UV)
4. Tungsten-Halogen Lamps
5. Light emitting diodes (LEDs) Multiple color LEDs can be bunched to provide a broad emission range)

Xenon Arc Lamp Profiles



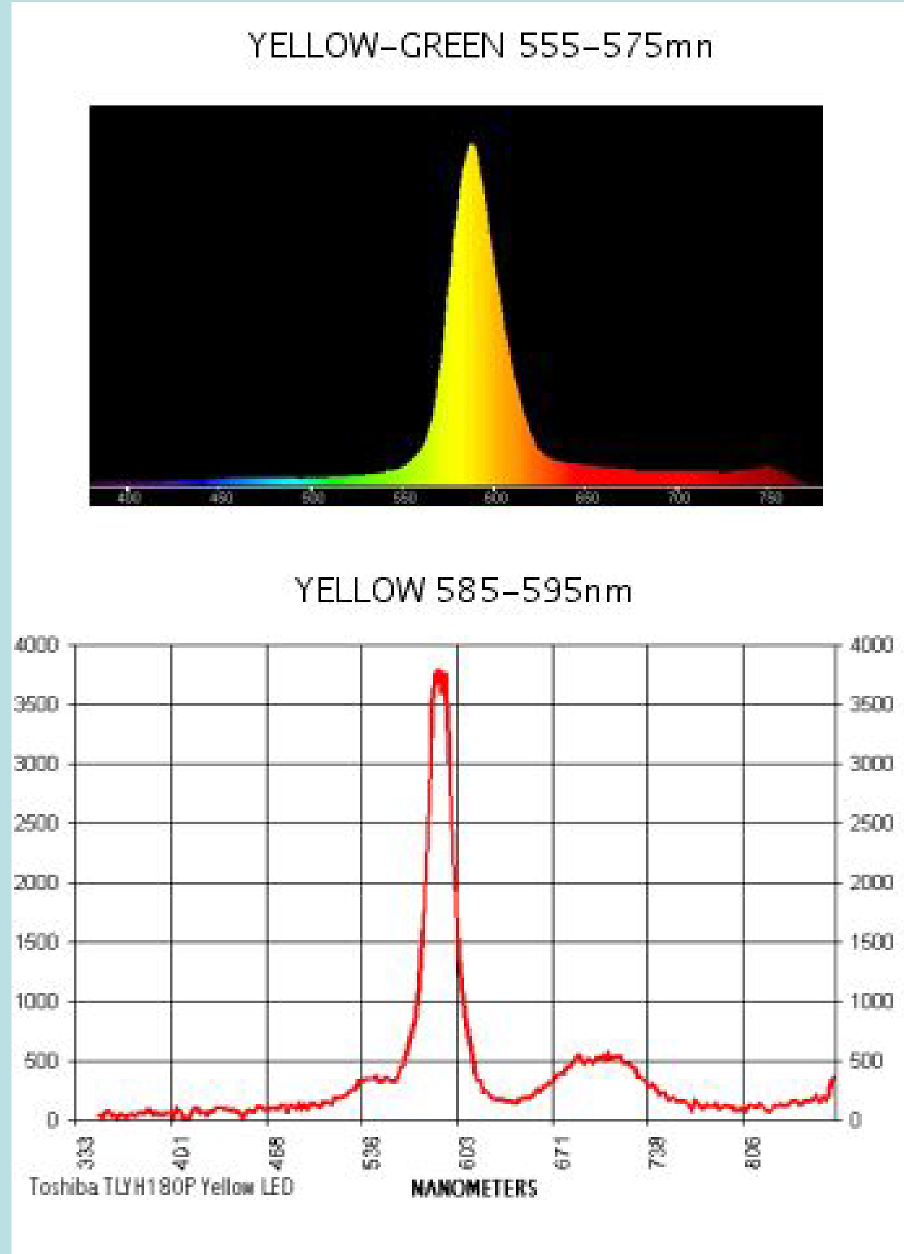
Mercury-Xenon Arc Lamp Profile



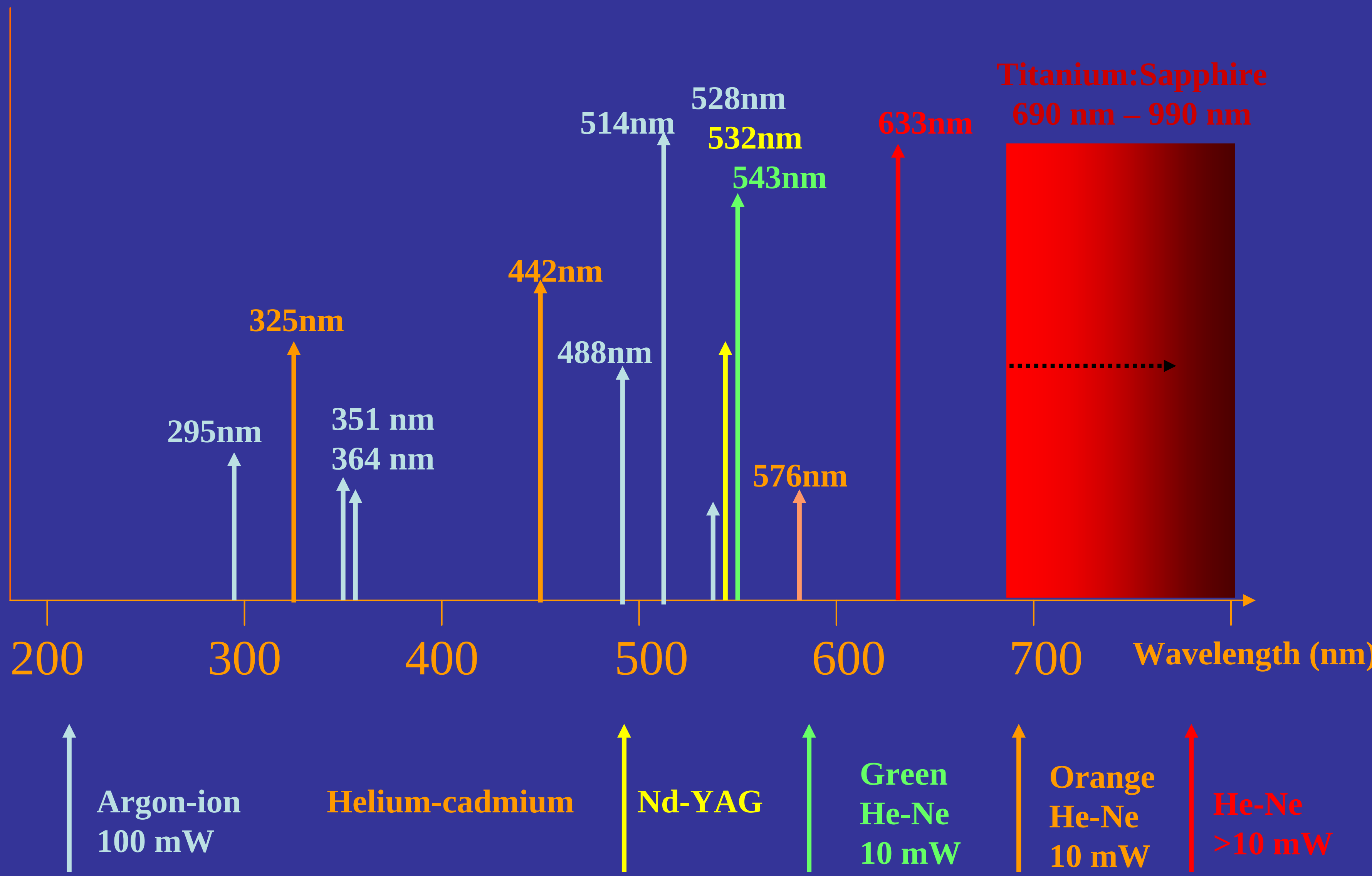
Light Emitting Diodes (LED)

Wavelengths from 350 nm to 1300 nm

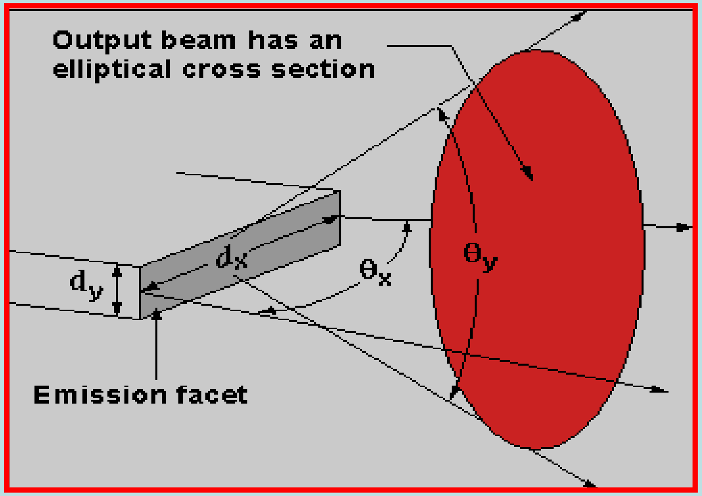
Near UV LED

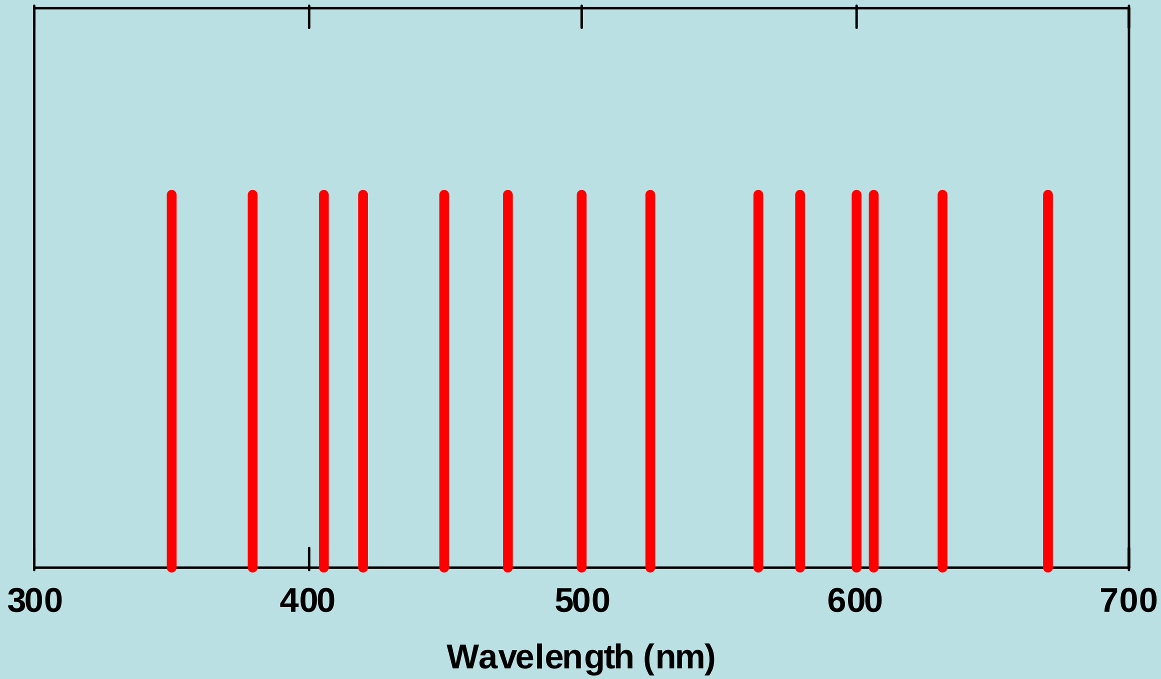


Lasers Light Sources

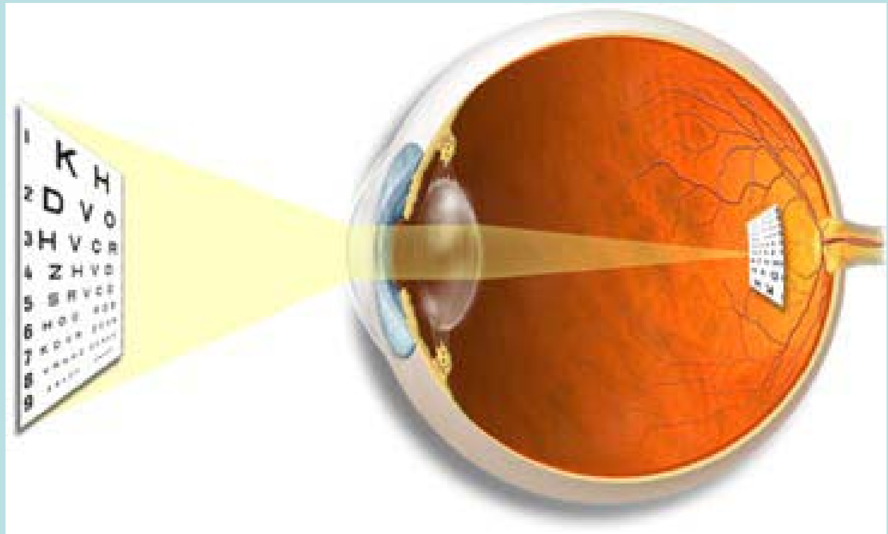


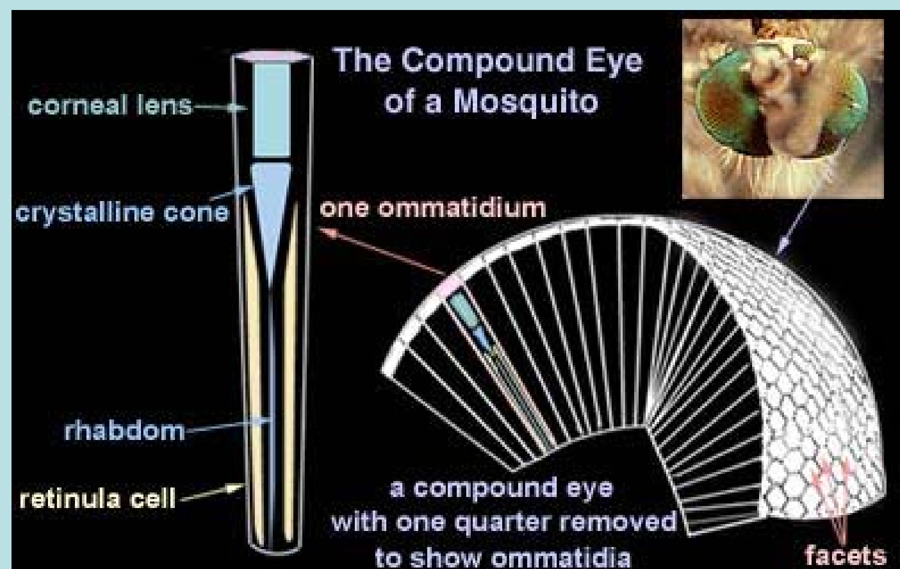
Laser Diodes





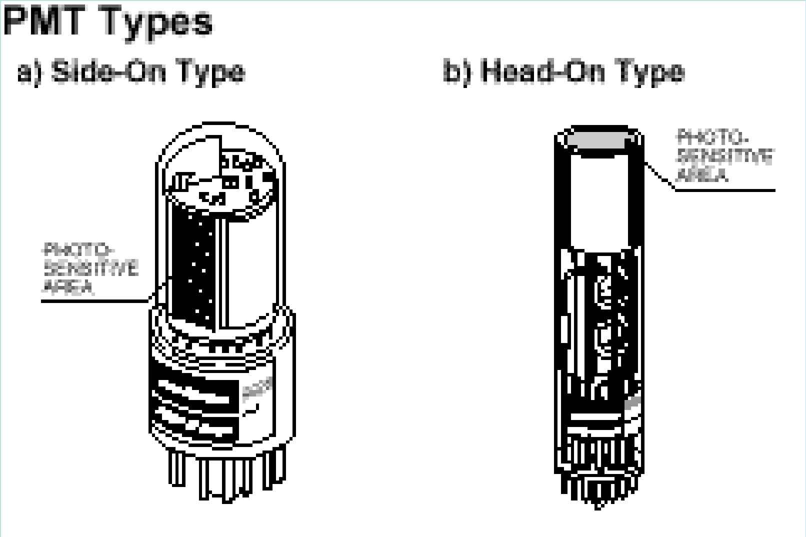
Detectors

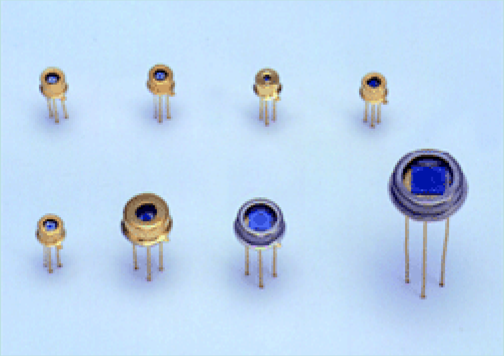




From http://www.eyedesignbook.com/index.html

Image courtesy of BioMEDIA ASSOCIATES http://www.ebiomedia.com



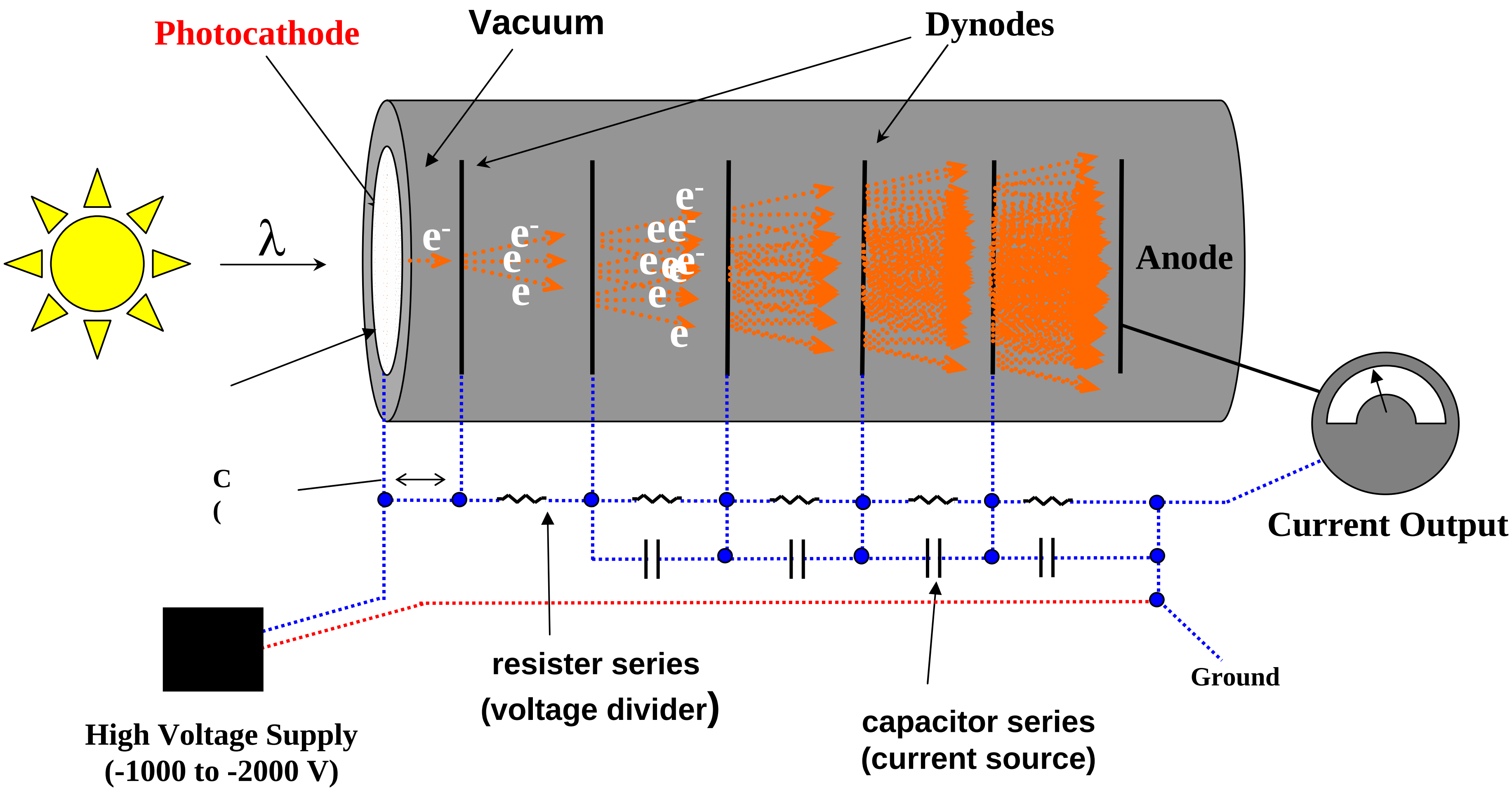


APD

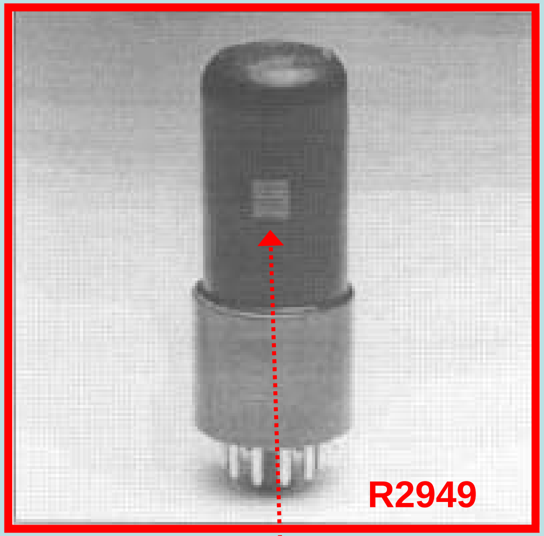
The silicon avalanche photodiode (Si APD) has a fast time response and high sensitivity in the near infrared region. APDs can be purchased from Hamamatsu with active areas from 0.2 mm to 5.0 mm in diameter and low dark currents (selectable). *Photo courtesy of Hamamatsu*

MCP & Electronics
(ISS Inc. Champaign, IL USA)

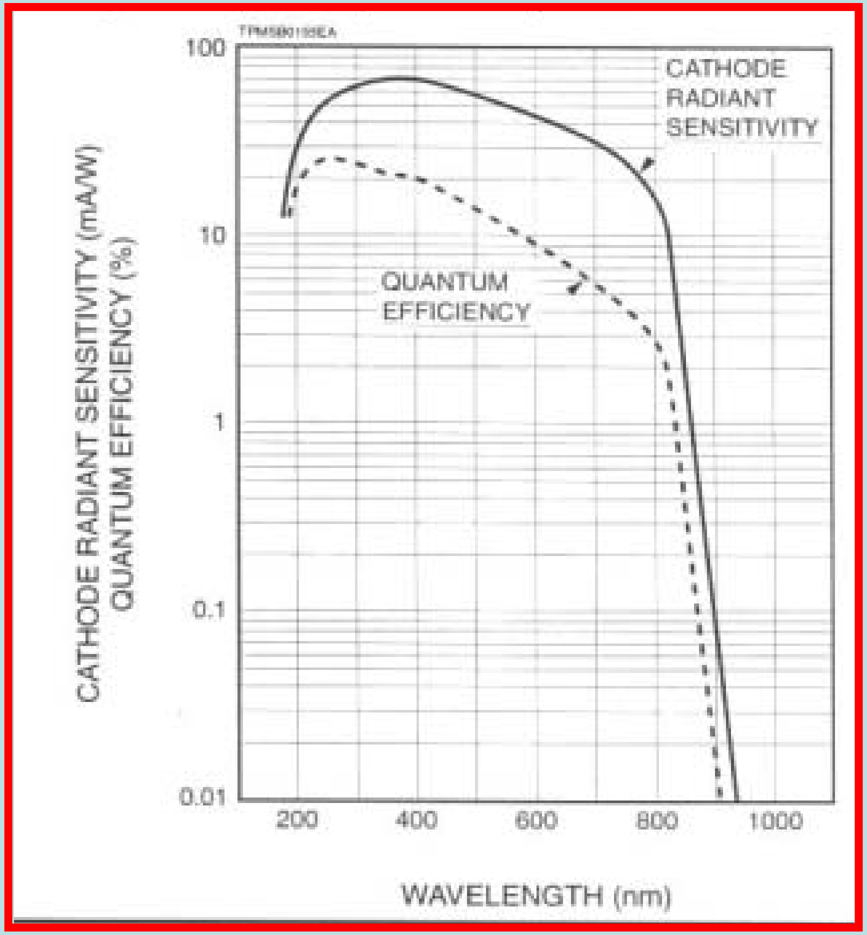
The Classic PMT Design



Hamamatsu R928 PMT Family

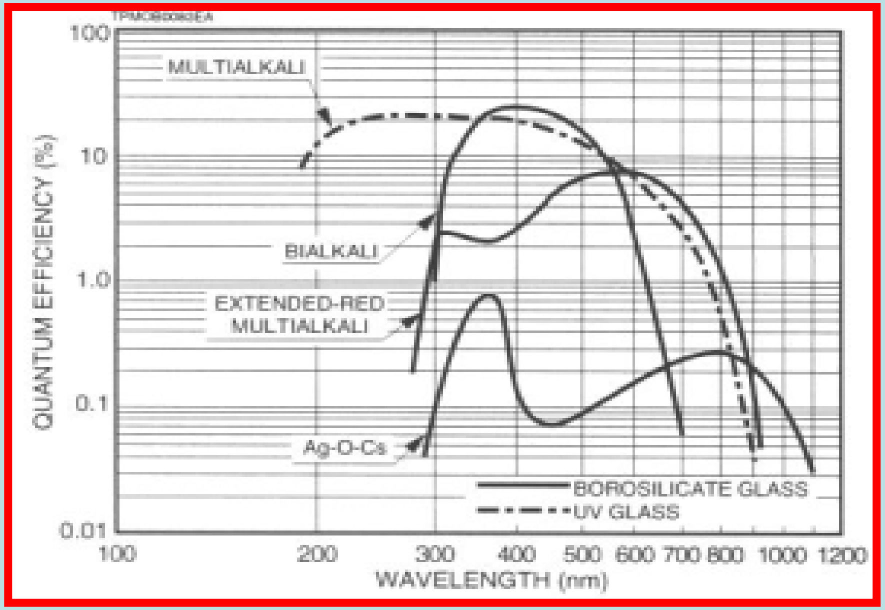


Window with Photocathode Beneath

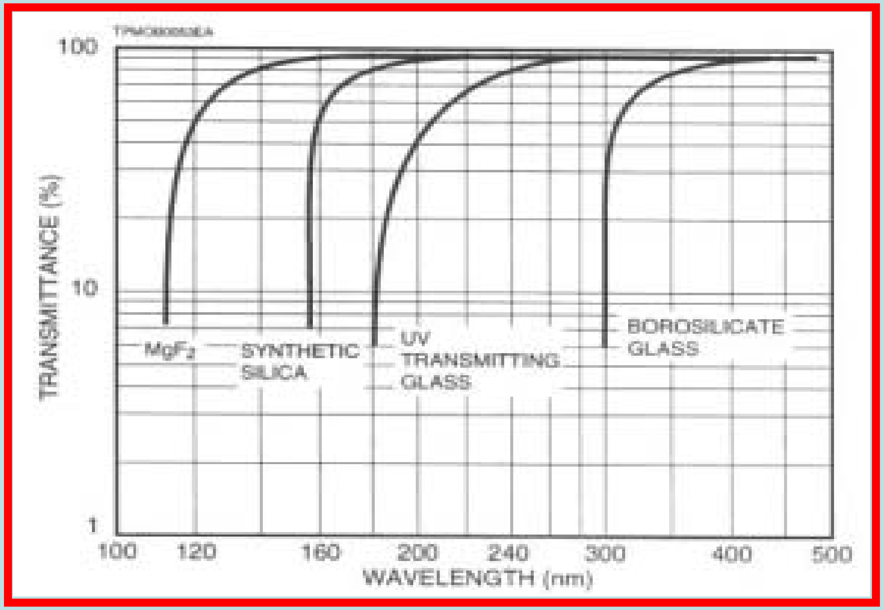


PMT Quantum Efficiencies

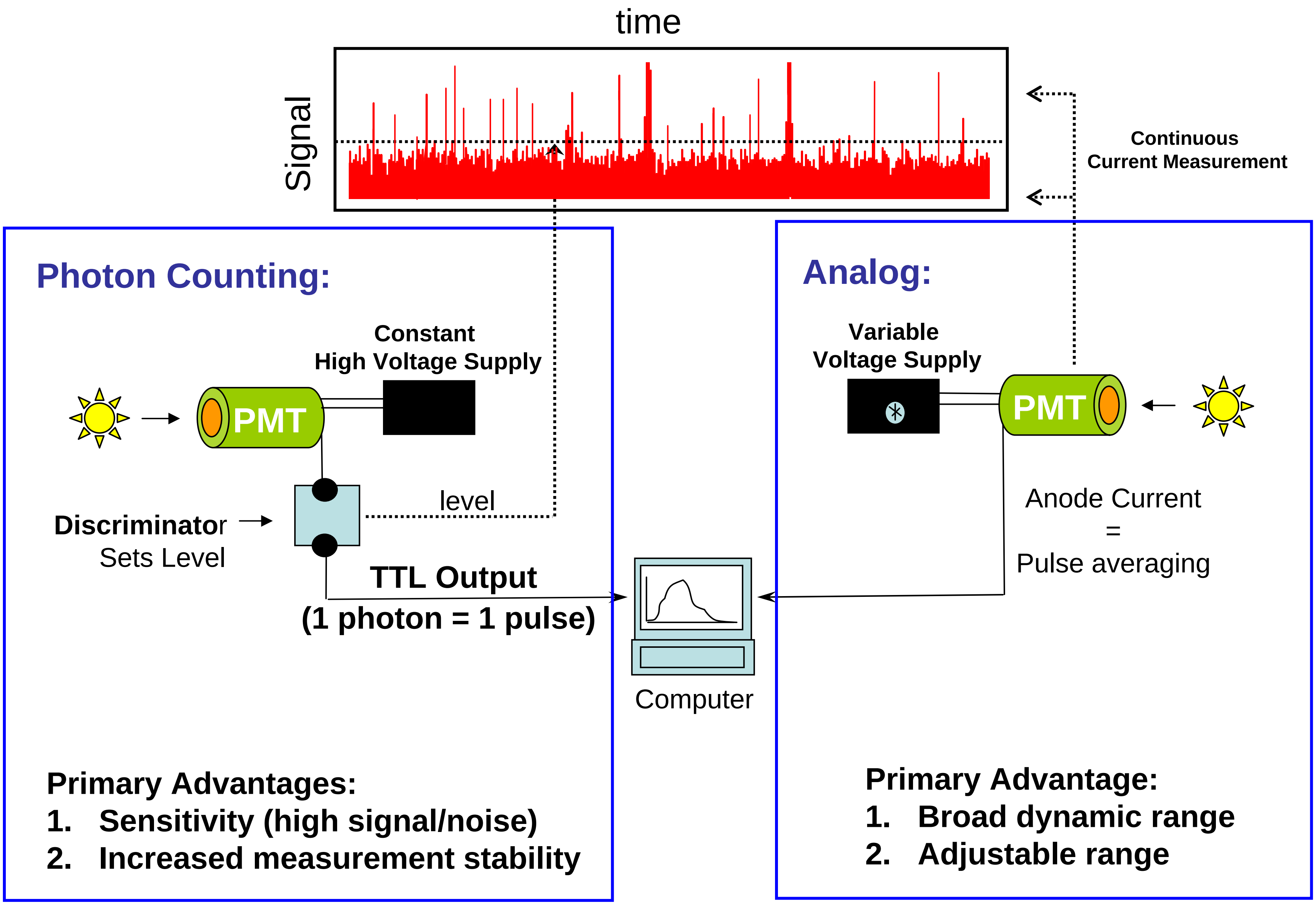
Cathode Material



Window Material



Photon Counting (Digital) and Analog Detection

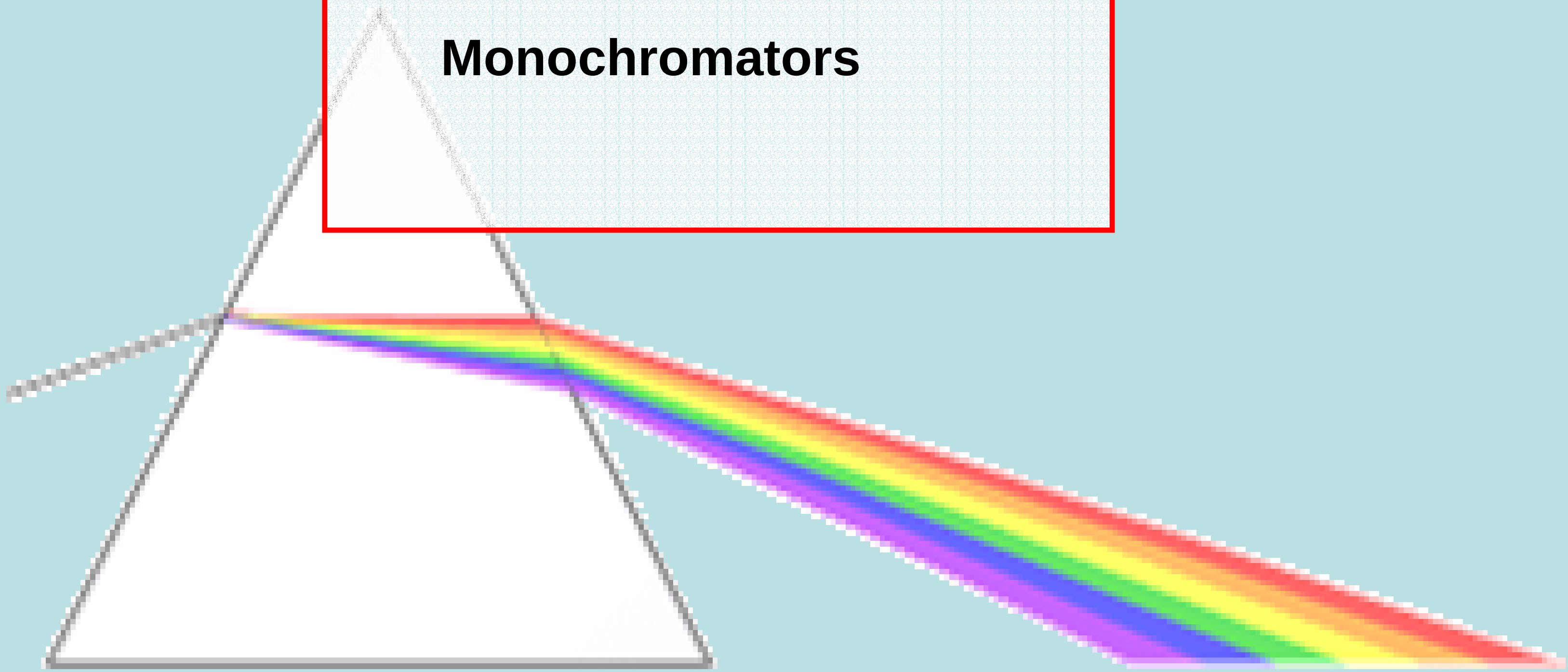


Wavelength Selection

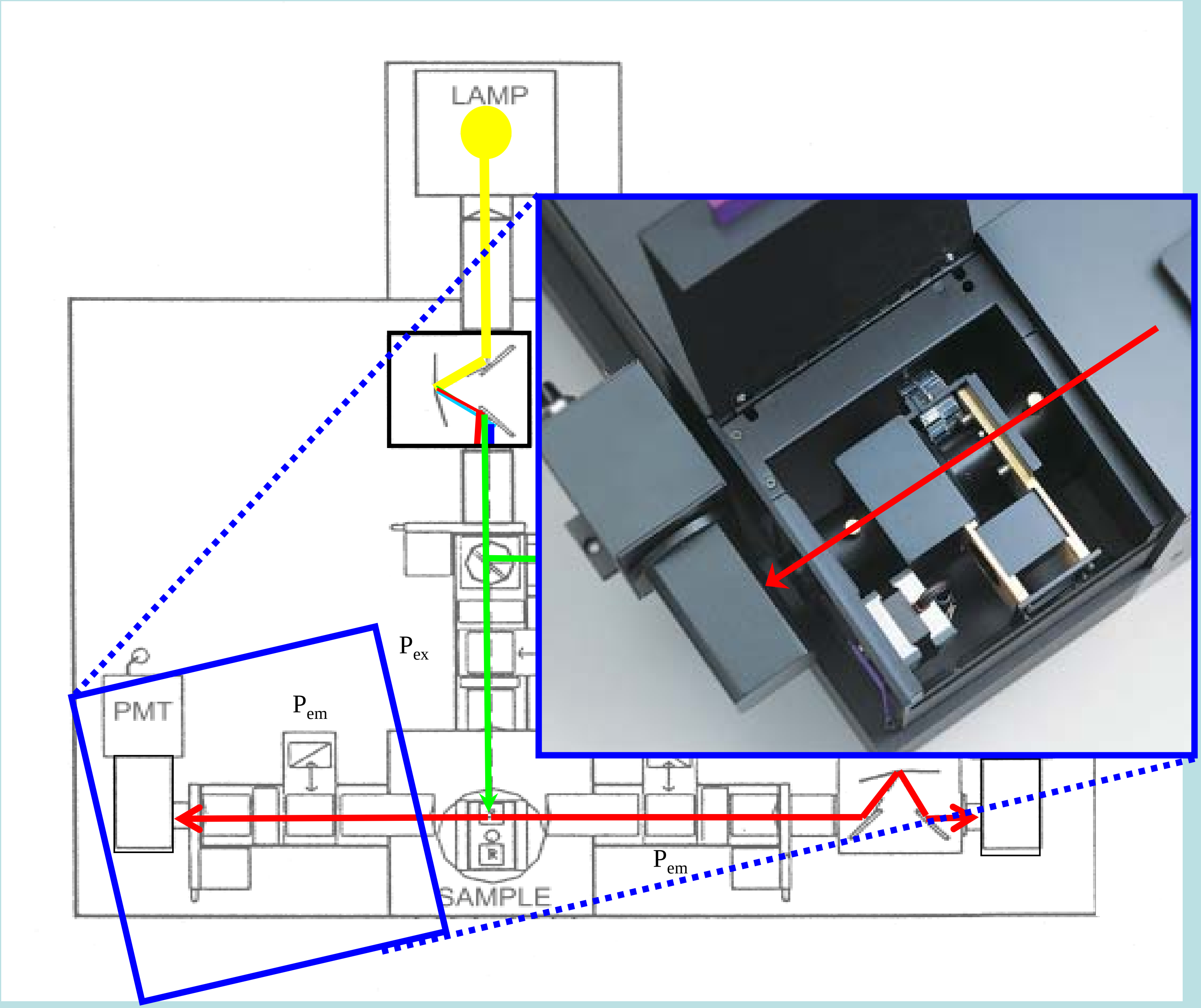
Fixed Optical Filters

Tunable Optical Filters

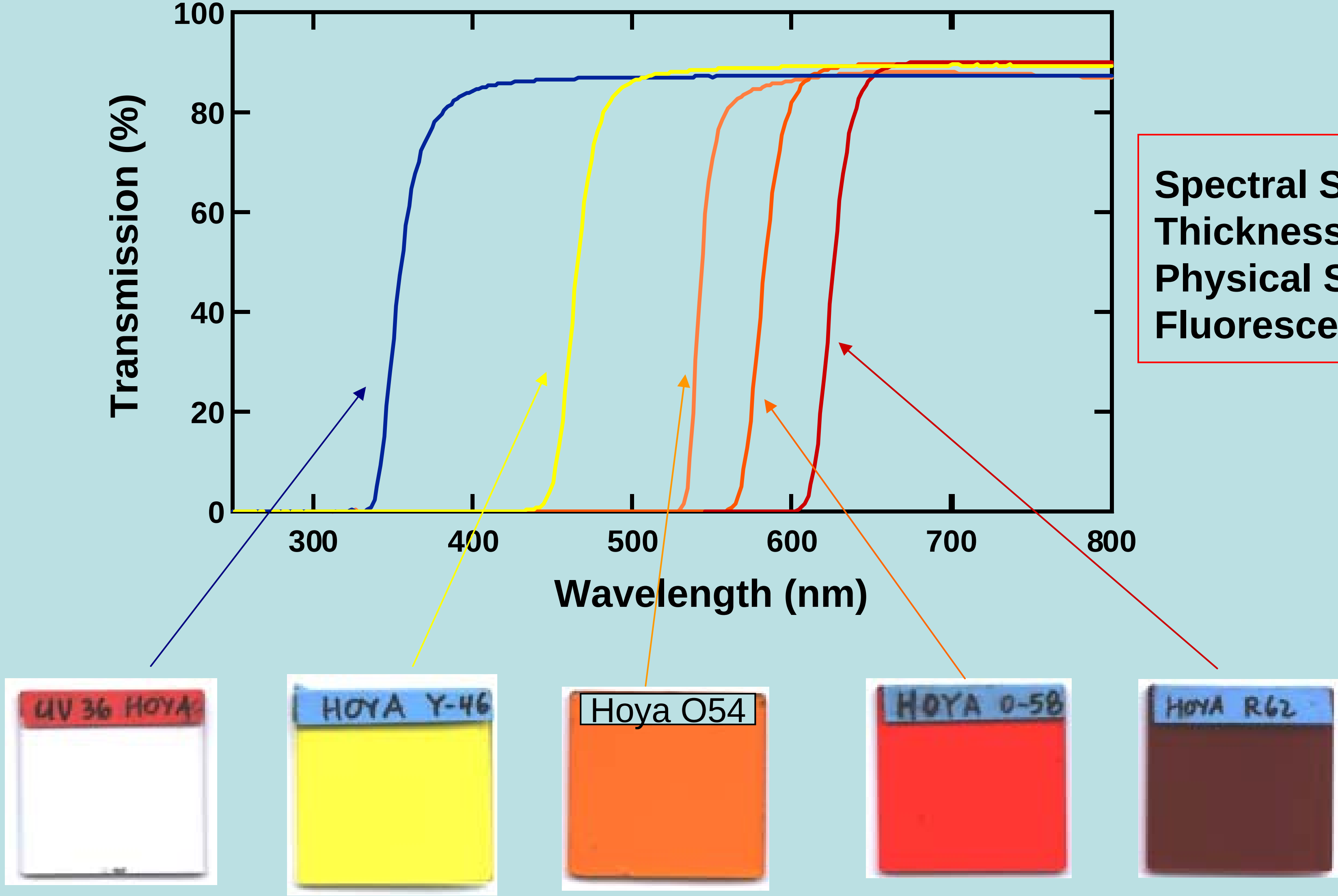
Monochromators



Optical Filter Channel

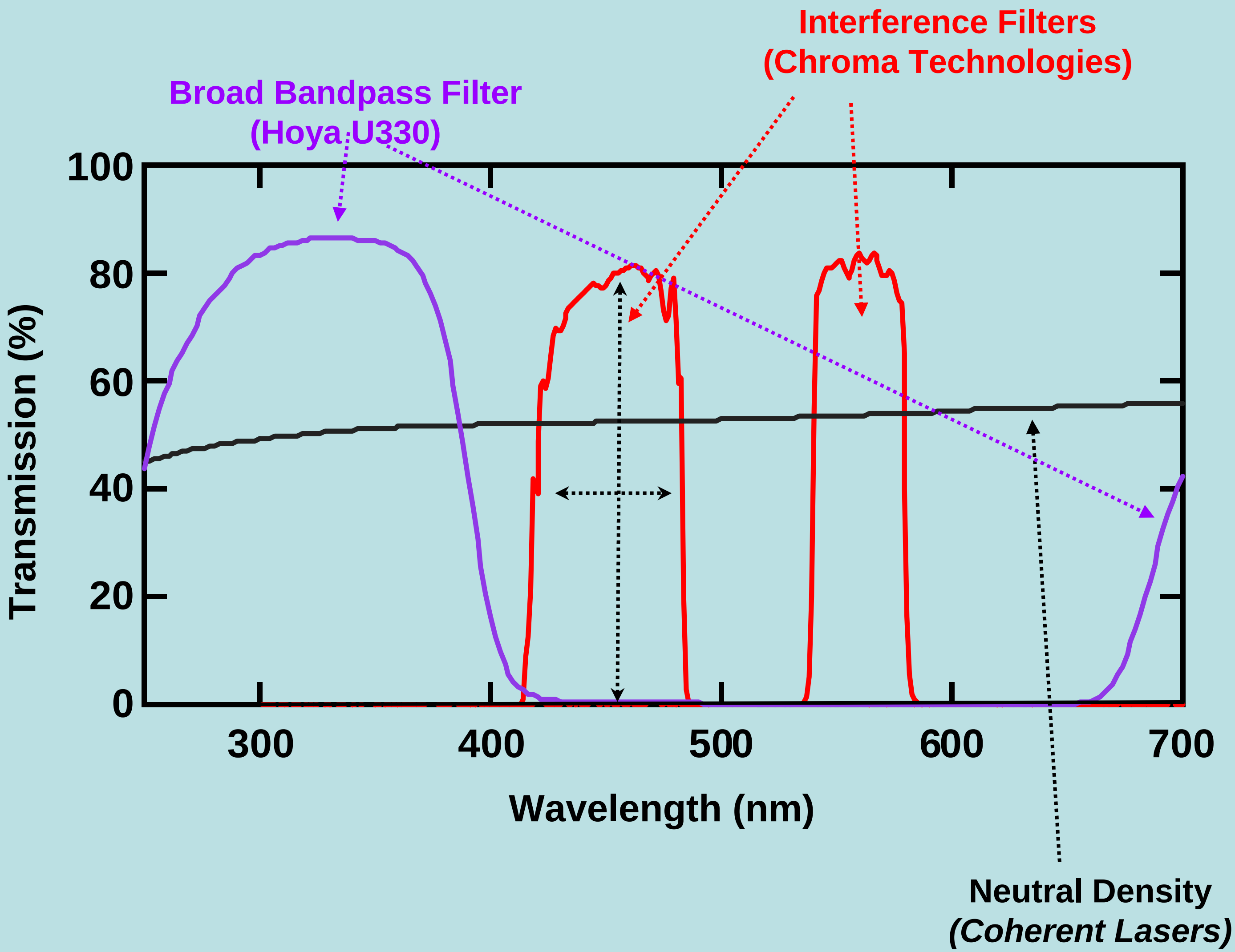


Long Pass Optical Filters



Spectral Shape
Thickness
Physical Shape
Fluorescence (!?)

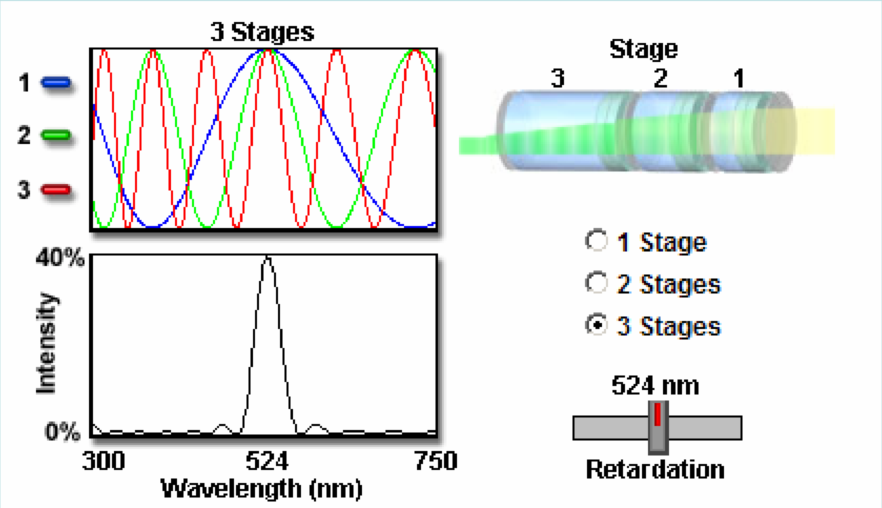
More Optical Filter Types…



Tunable Optical Filters

Liquid Crystal Filters:

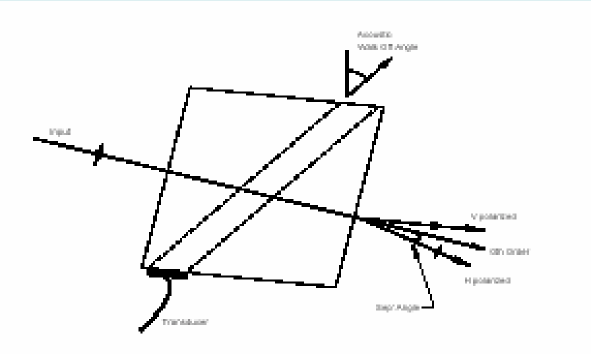
An electrically controlled liquid crystal elements to select a specific visible wavelength of light for transmission through the filter at the exclusion of all others.



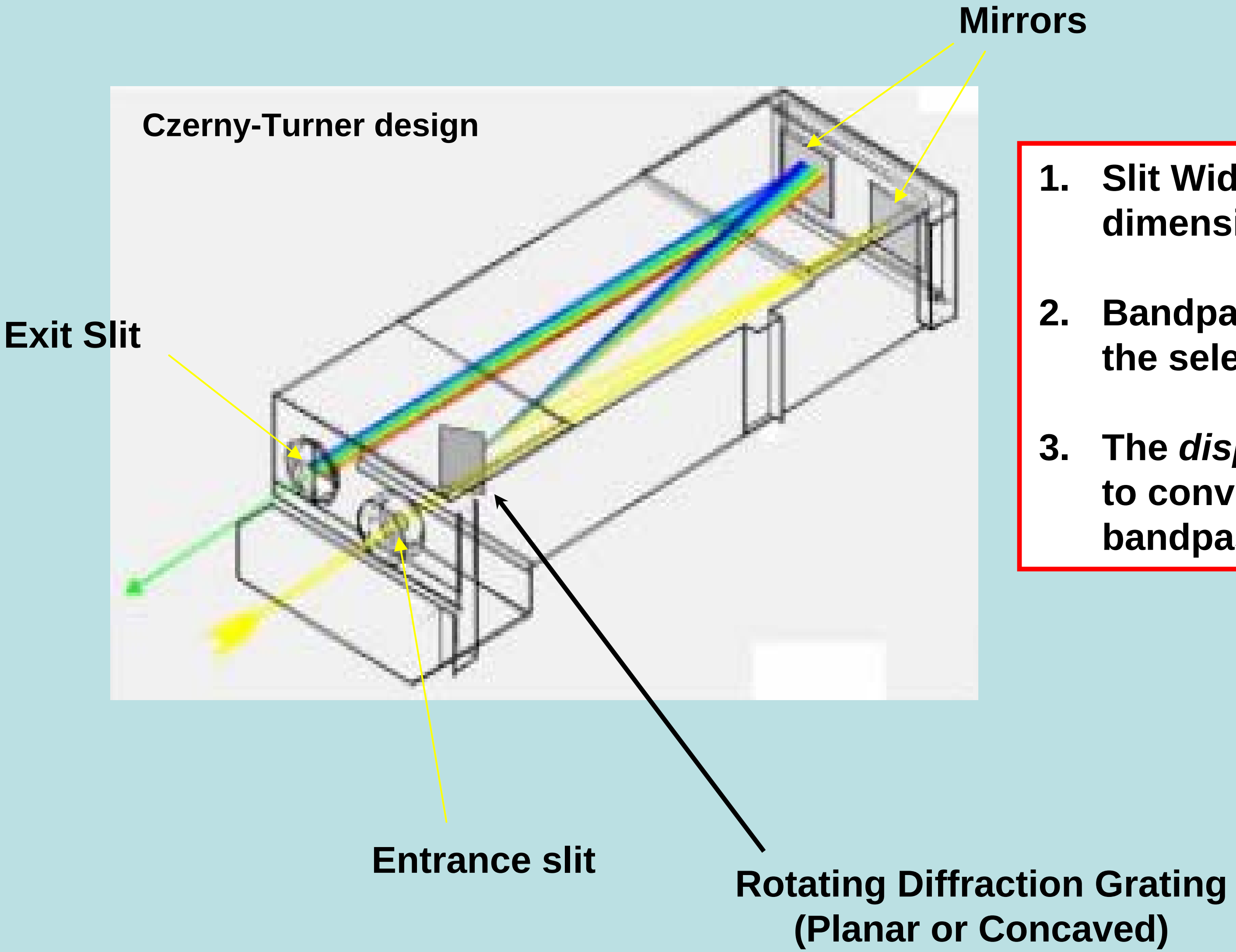
AO Tunable Filters:

The AOTF range of acousto-optic devices are solid state optical filters. The wavelength of the diffracted light is selected according to the frequency of the RF drive signal.

Isomet (http://www.isomet.com/index.html)



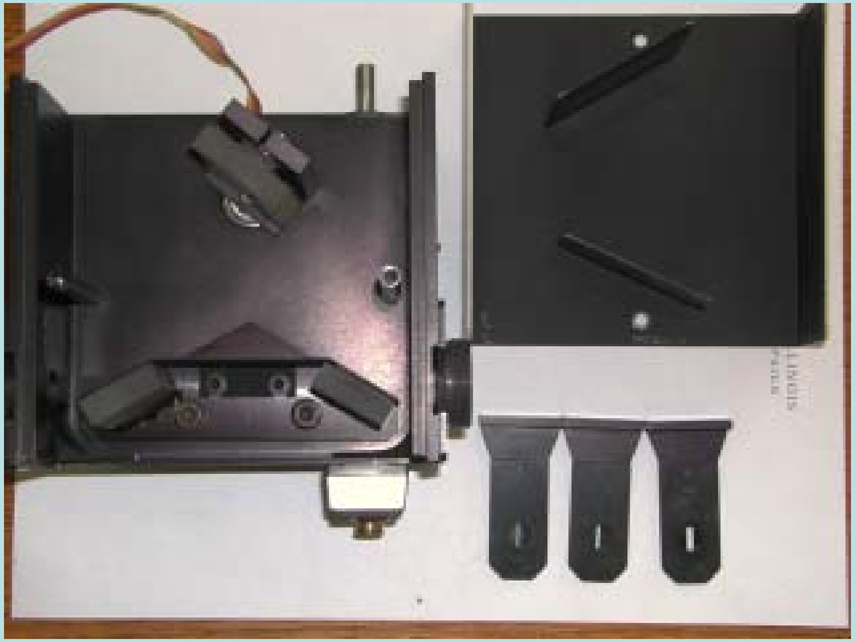
Monochromators

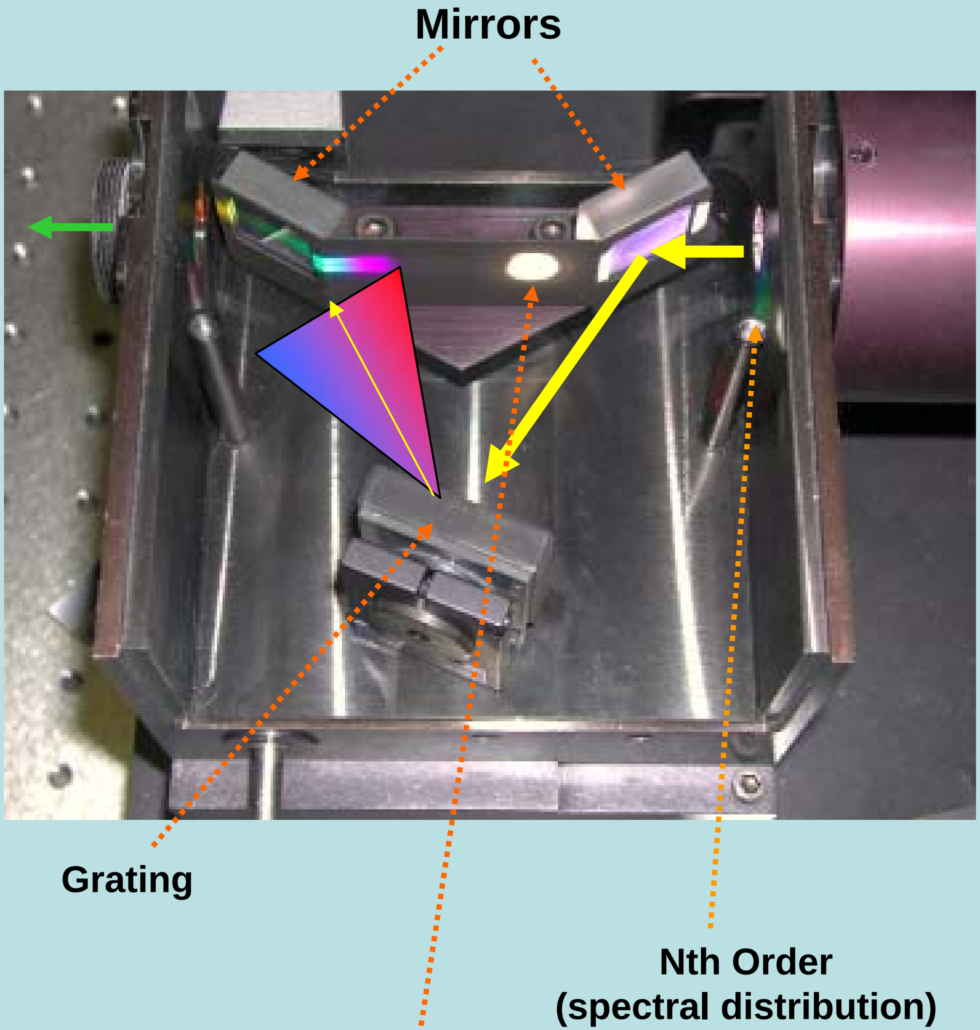


1. Slit Width (mm) is the dimension of the slits.
2. Bandpass is the FWHM of the selected wavelength.
3. The *dispersion* is the factor to convert slit width to bandpass.

The Inside of a Monochromator

H10 Monochromator



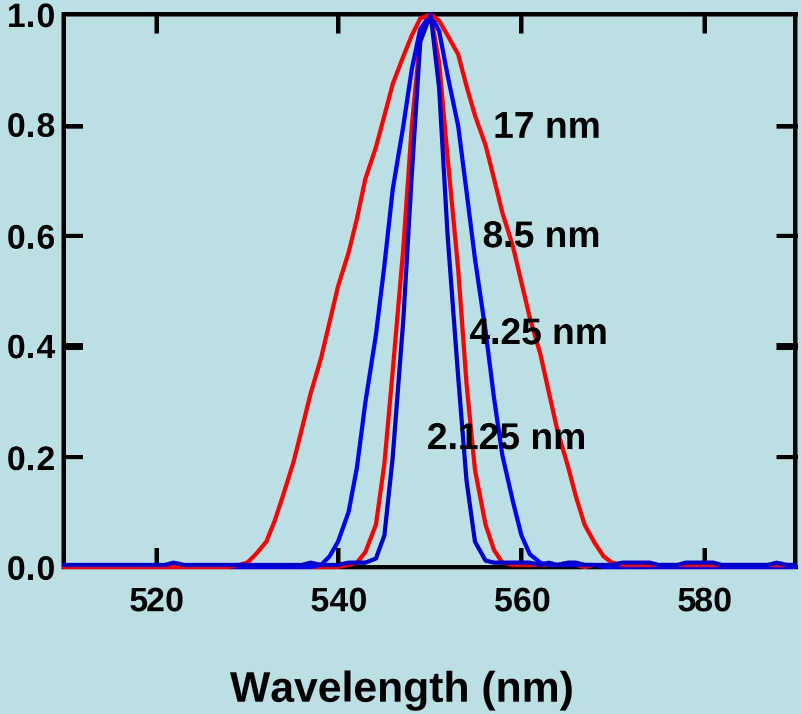


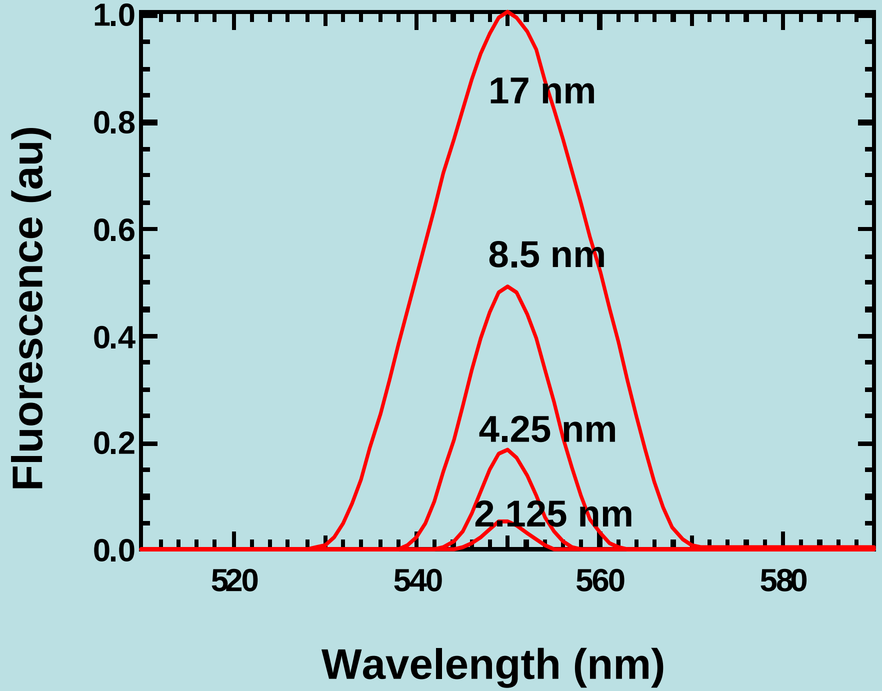
Changing the Bandpass

1. Drop in intensity
2. Narrowing of the spectral selection

Fixed Excitation Bandpass = 4.25 nm

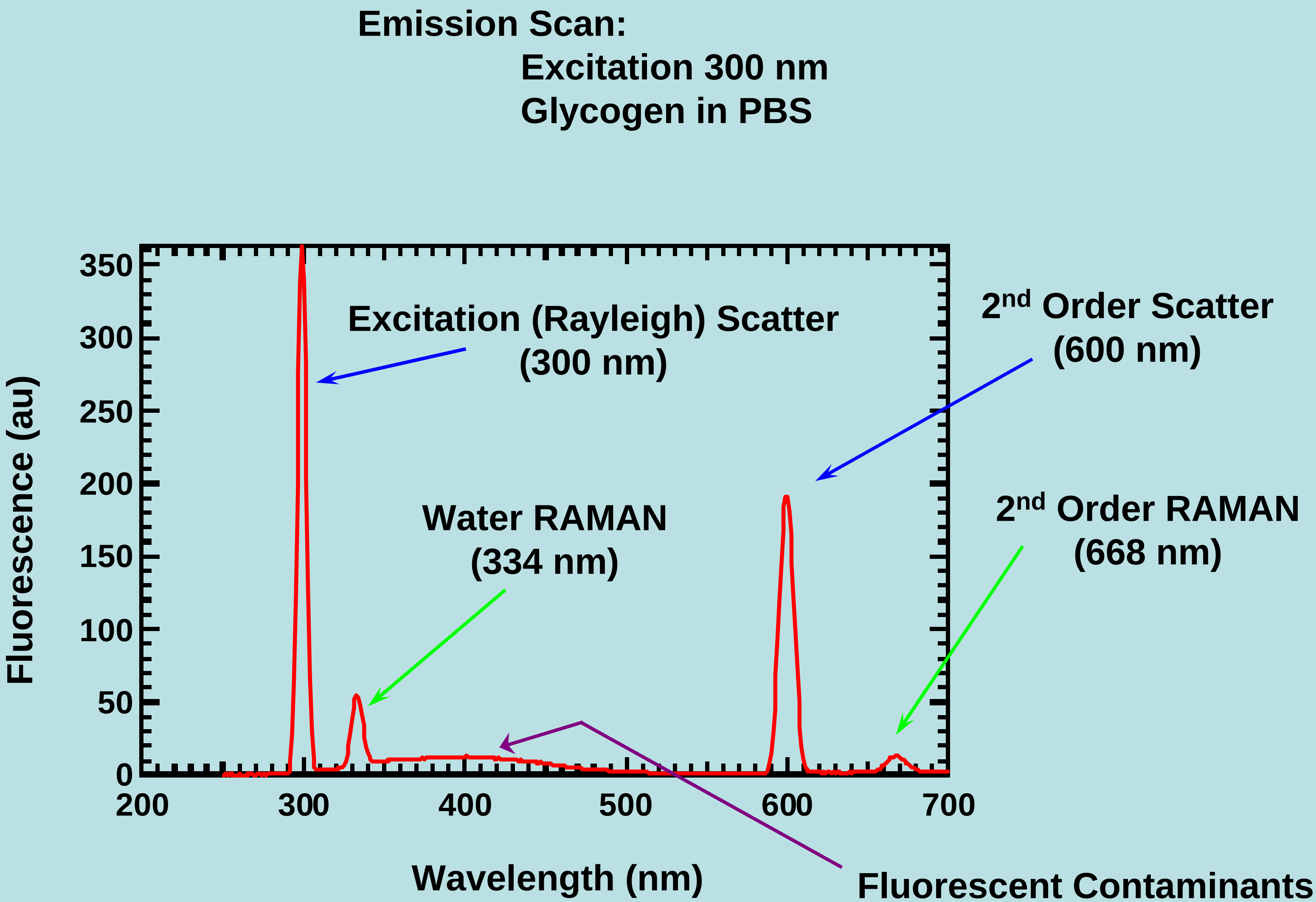
Changing the Emission Bandpass





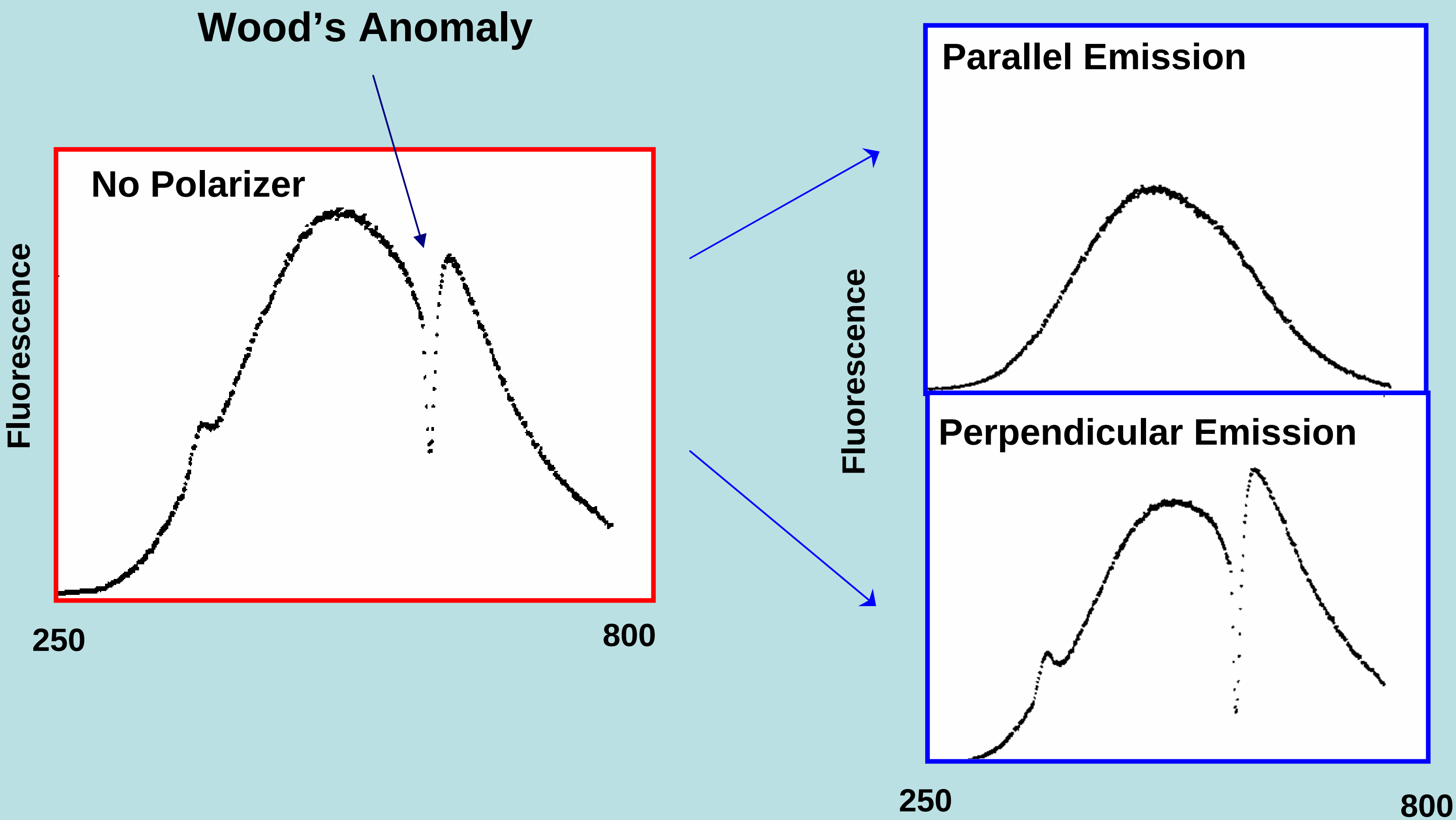
Collected on a SPEX Fluoromax - 2

Higher Order Light Diffraction



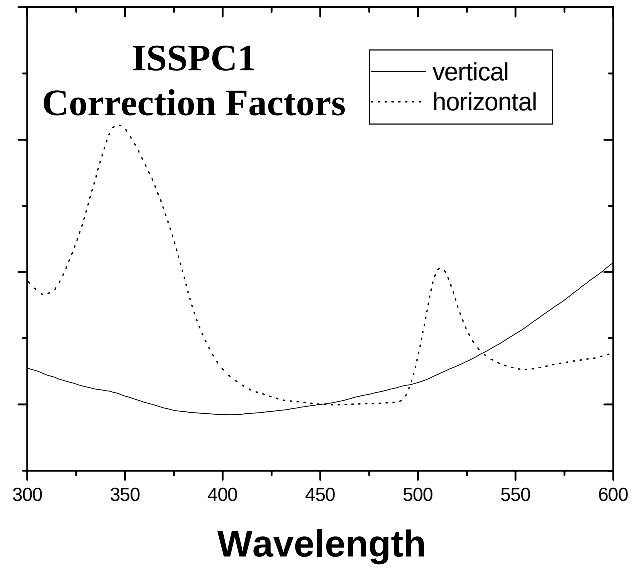
Monochromator Polarization Bias

Tungsten Lamp Profile Collected on an SLM Fluorometer

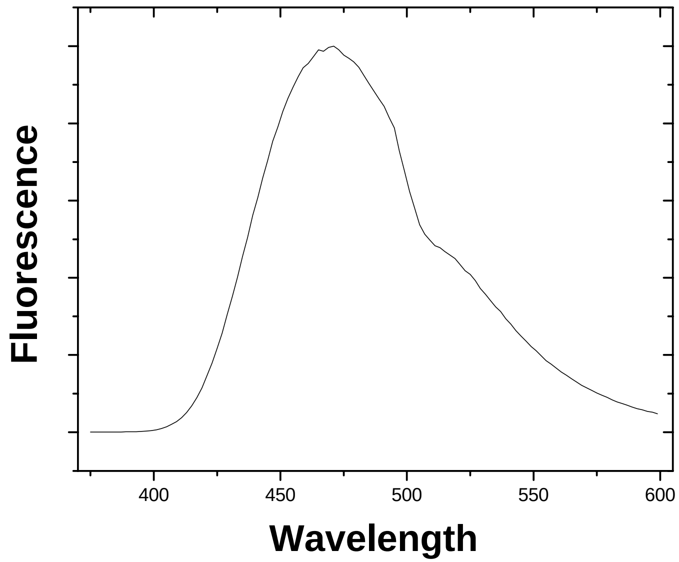


Adapted from Jameson, D.M., Instrumental Refinements in Fluorescence Spectroscopy: Applications to Protein Systems., in Biochemistry, Champaign-Urbana, University of Illinois, 1978.

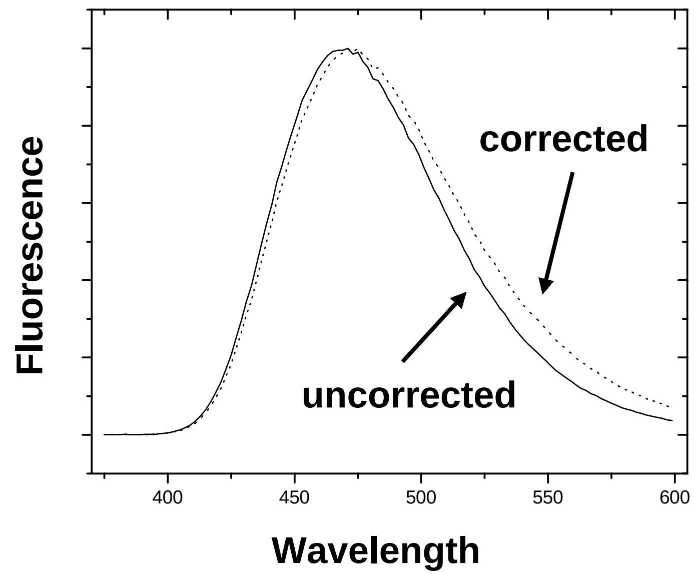
Correction of Emission Spectra



ANS Emission Spectrum, no polarizer

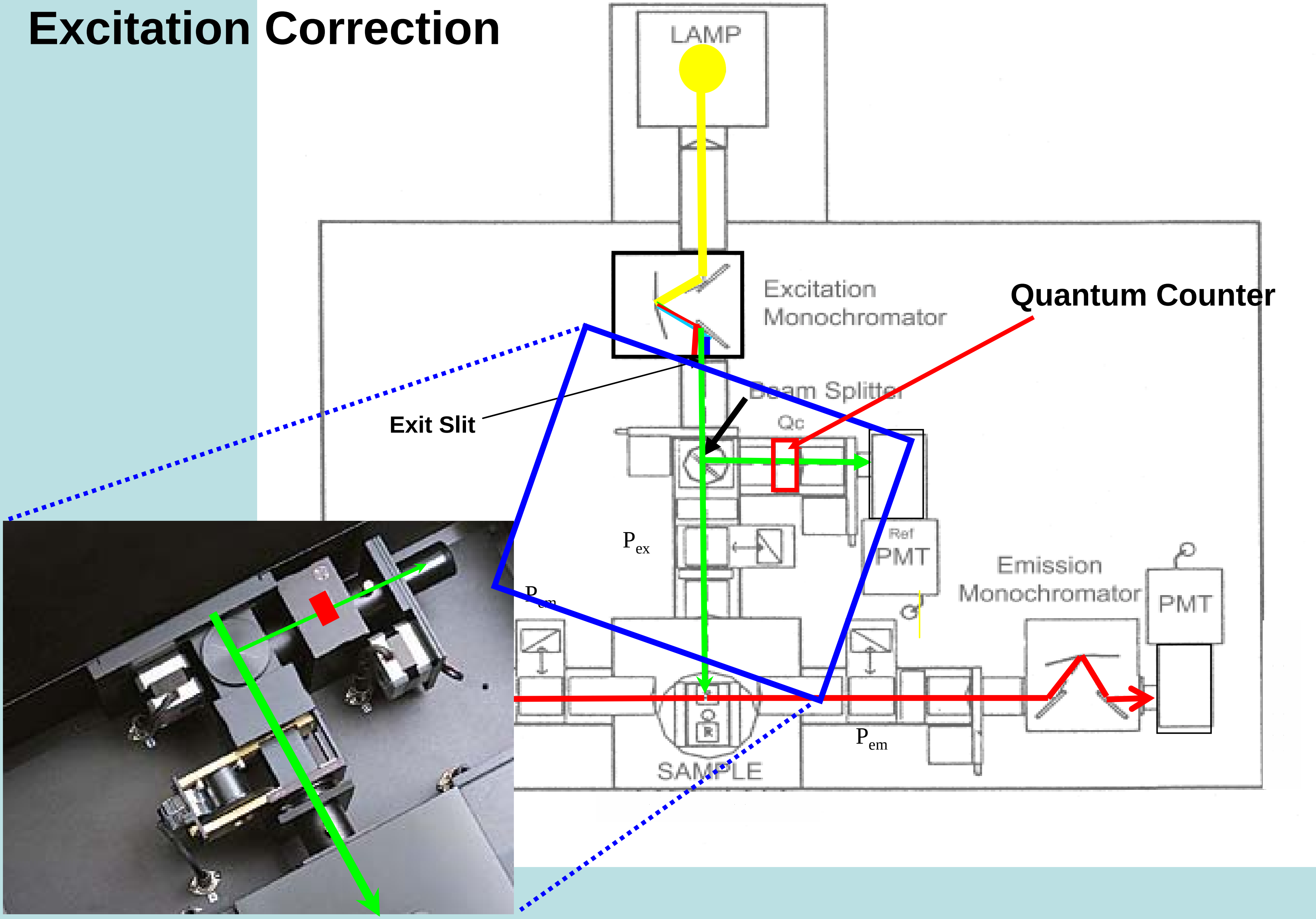


ANS Emission Spectrum, parallel polarizer



from Jameson et. Al., Methods in Enzymology, 360:1

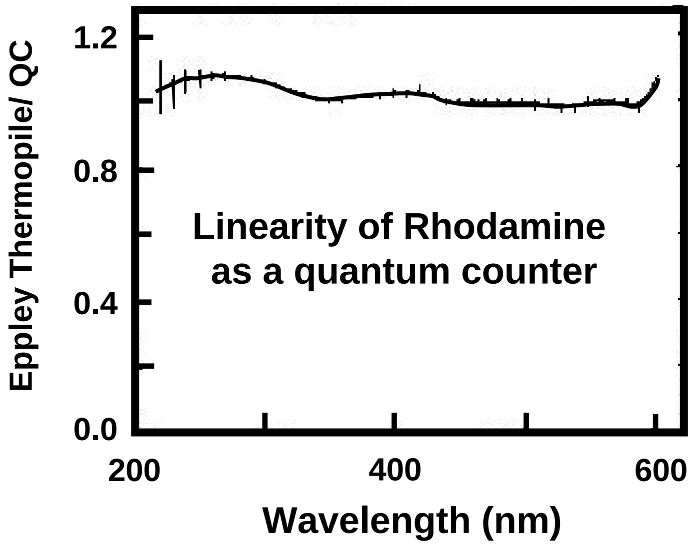
Excitation Correction

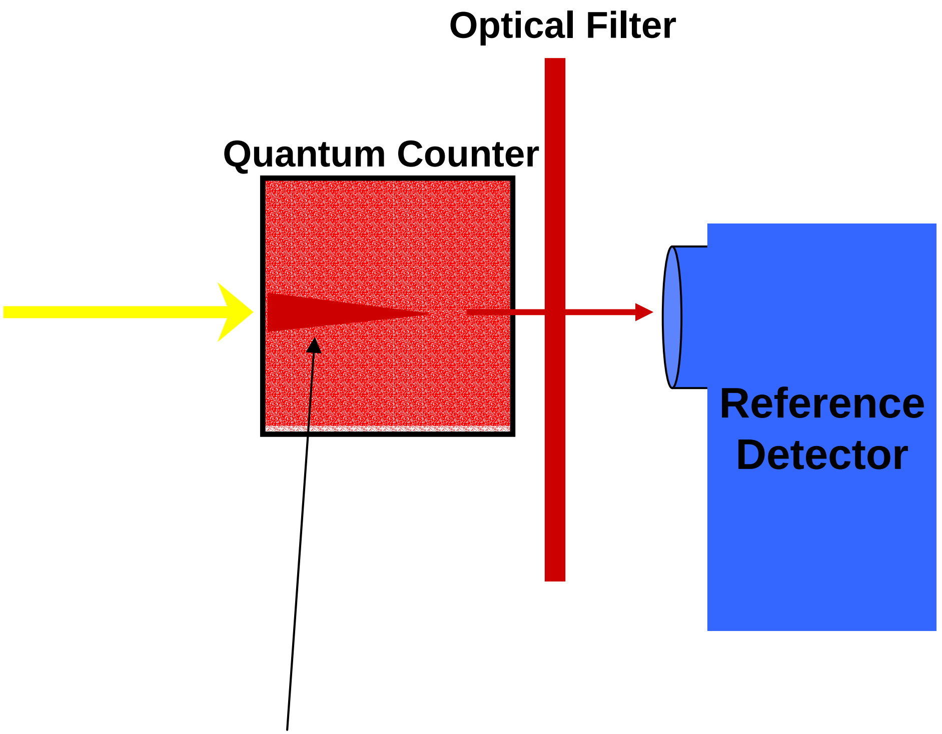


The Instrument Quantum Counter

Common Quantum Counters (optimal range)*

Rhodamine B	**(220 - 600 nm)**
Fluorescein	**(240 - 400 nm)**
Quinine Sulfate	**(220 - 340 nm)**





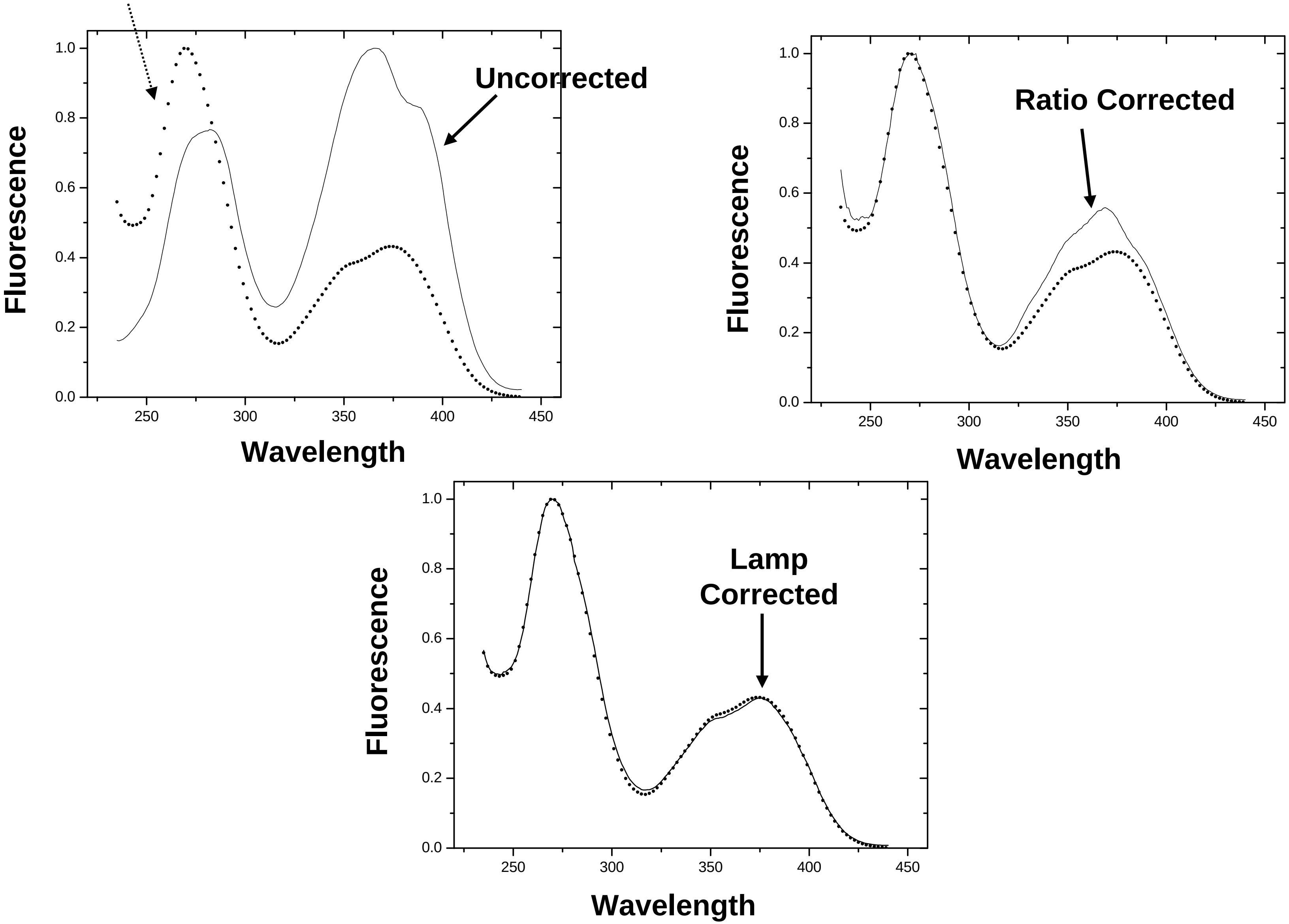
Fluorescence

Here we want the inner filter effect!

*** Melhuish (1962) J. Opt. Soc. Amer. 52:1256**

Excitation Correction

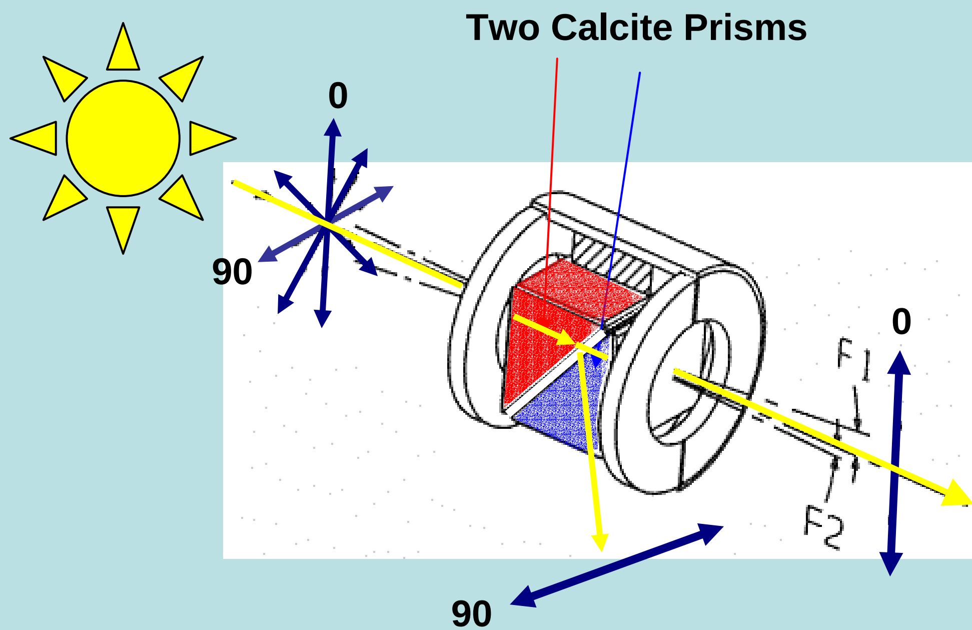
Absorption (dotted line) and Excitation Spectra (solid line) of ANS in Ethanol



from Jameson et. Al., Methods in Enzymology, 360:1

Polarizers

The *Glan Taylor* prism polarizer



Common Types:

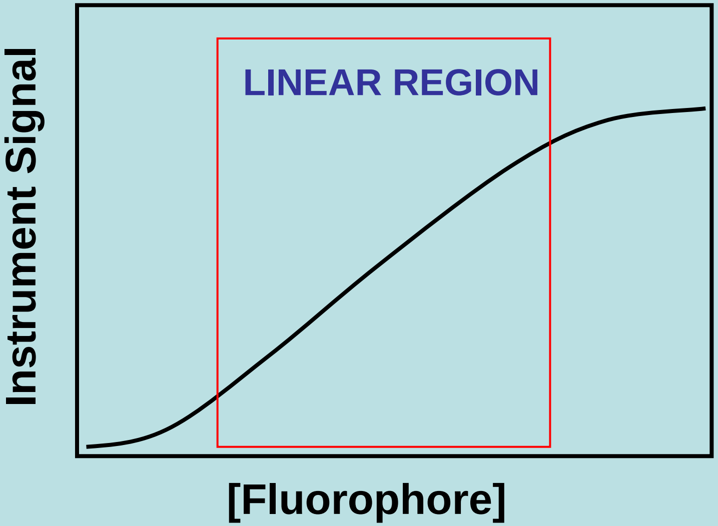
- **Glan Taylor (air gap)**
- **Glan Thompson**
- **Sheet Polarizers**

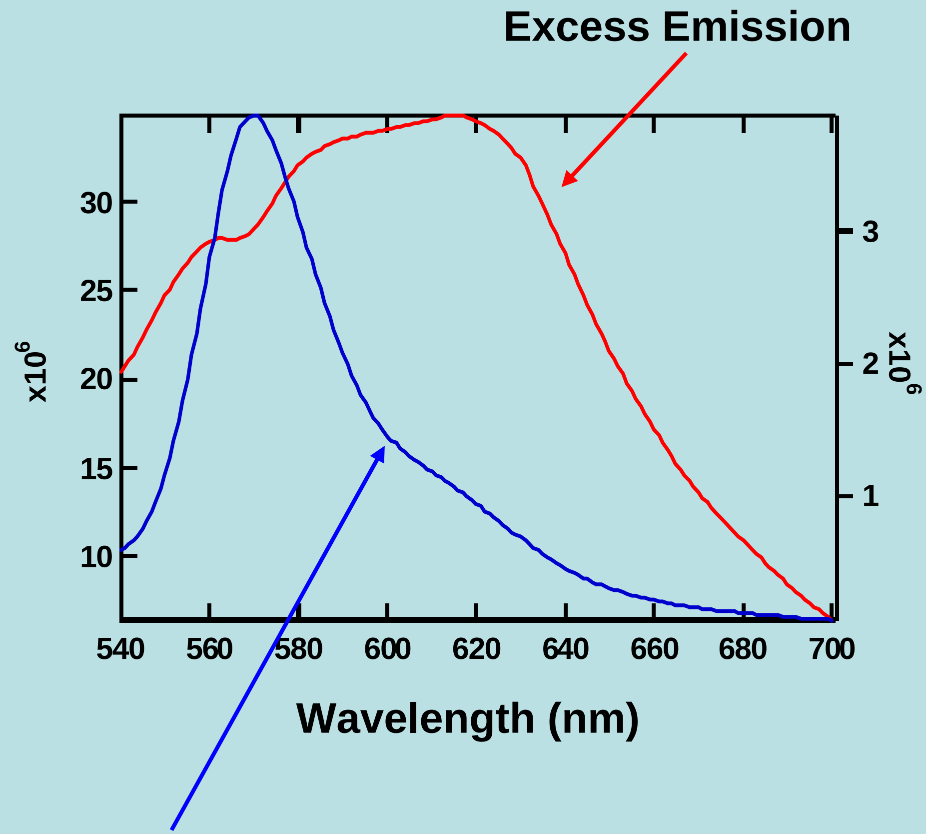
Two UV selected calcite prisms are assembled with an intervening air space. The calcite prism is birefringent and cut so that only one polarization component continues straight through the prisms. The spectral range of this polarizer is from 250 to 2300 nm. At 250 nm there is approximately 50% transmittance.

Sample Issues

Signal Attenuation of the Excitation Light
PMT Saturation

Fluorescence vs Signal



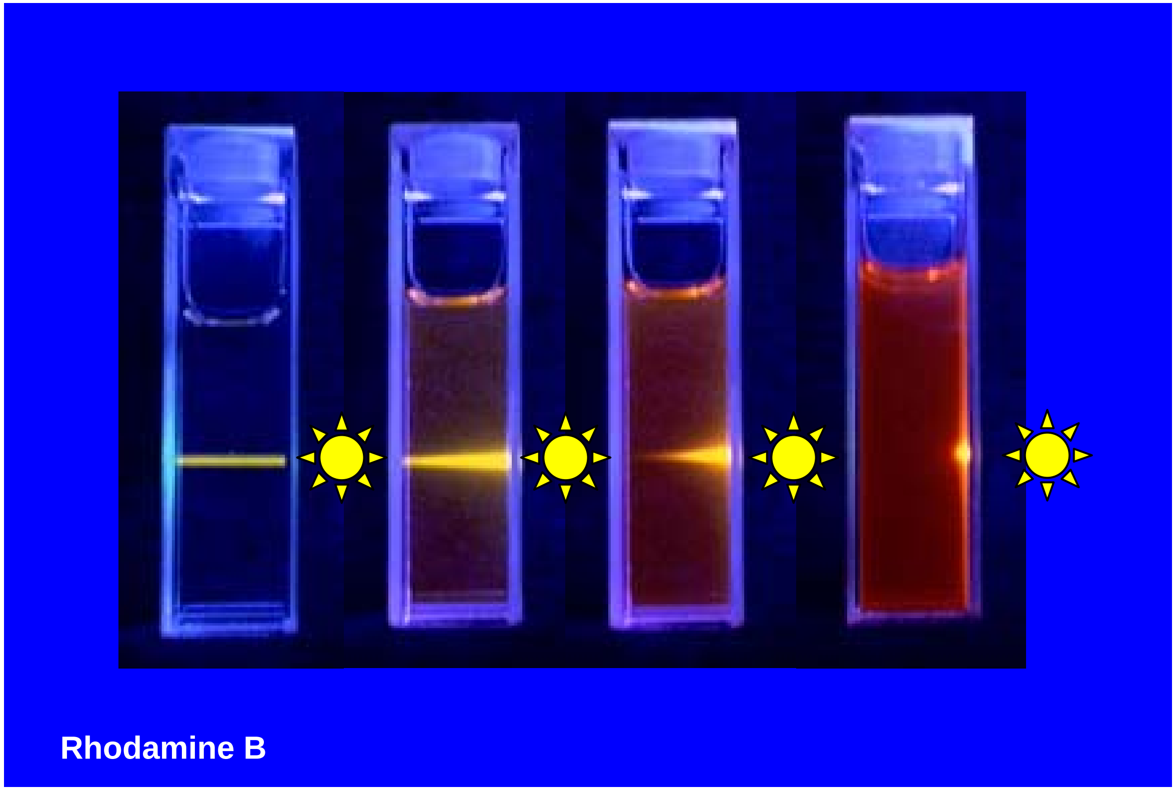


Reduced emission intensity

1. **ND Filters**
2. **Narrow slit widths**
3. **Move off absorbance peak**

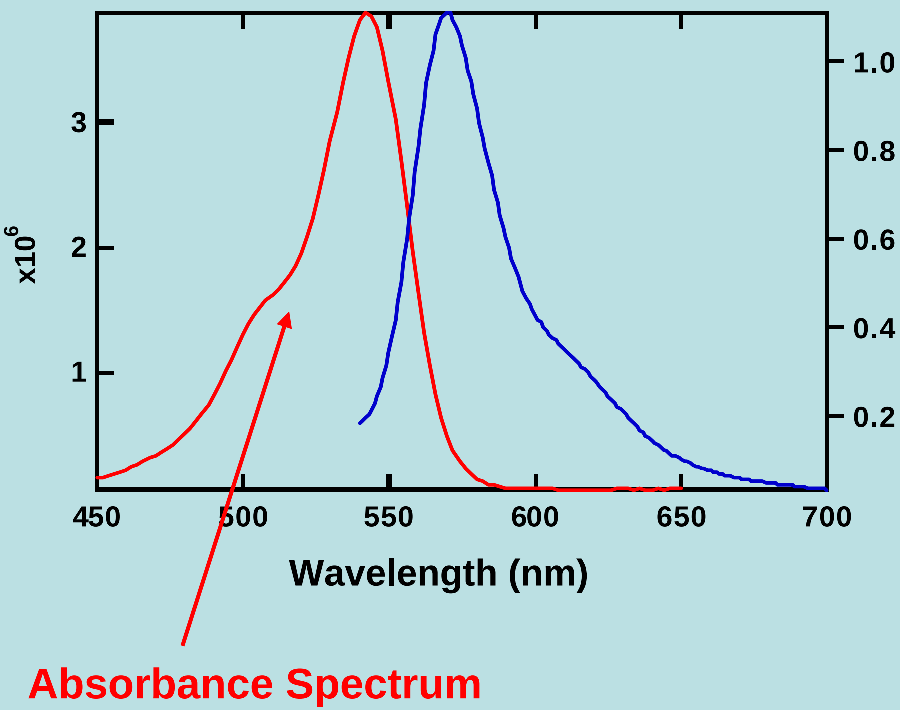
Attenuation of the Excitation Light through Absorbance

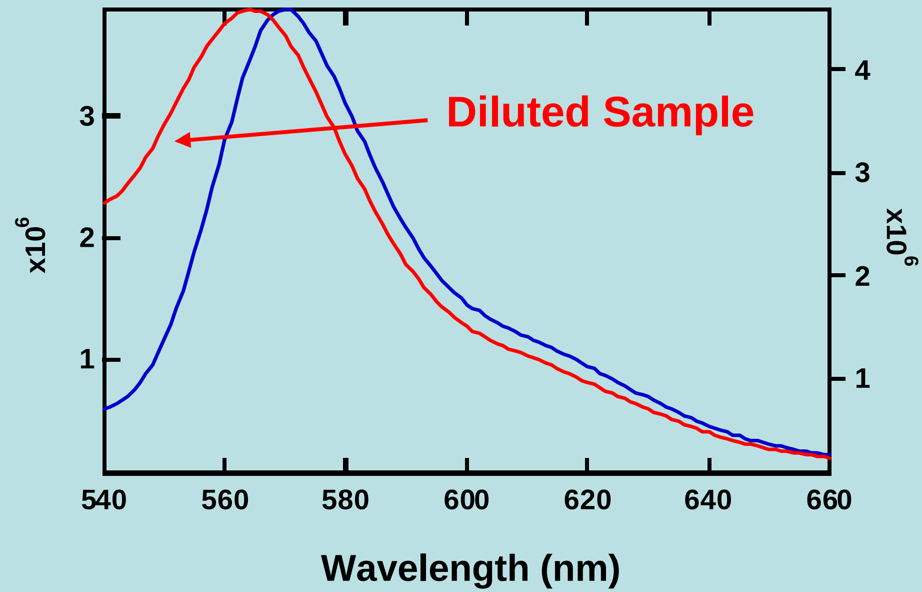
Sample concentration
& the *inner filter effect*



from Jameson et. al., Methods in Enzymology (2002), 360:1

The second half of the *inner filter effect*: attenuation of the emission signal.

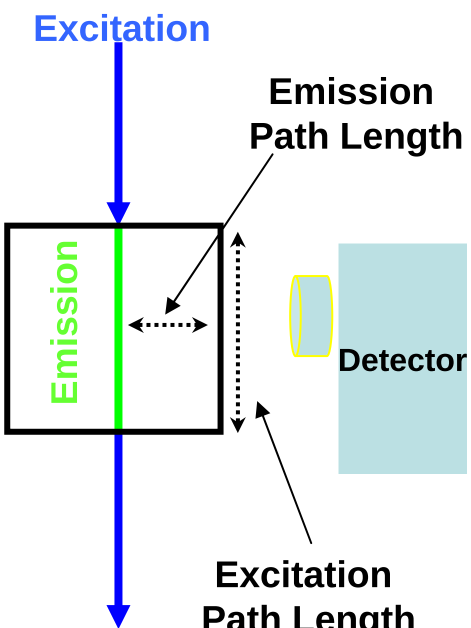


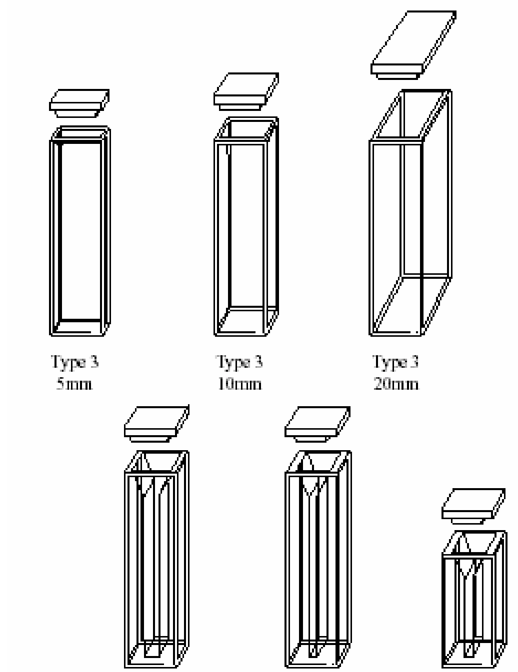


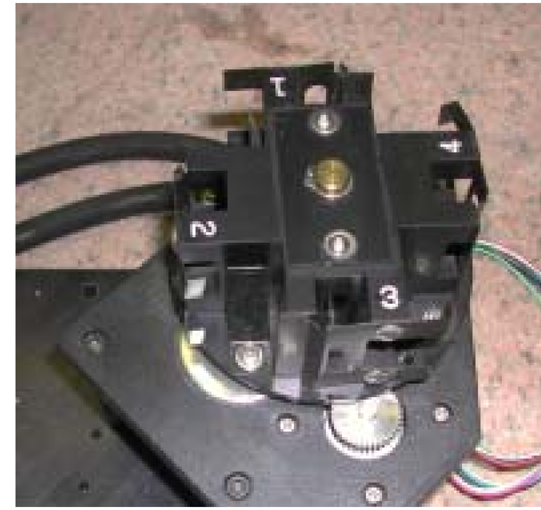
(1) Spectral Shift
(2) Change in Spectral Shape

How do we handle highly absorbing solutions?

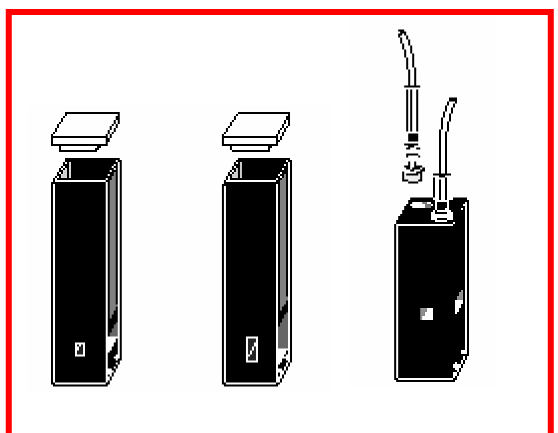
Quartz/Optical Glass/Plastic Cells

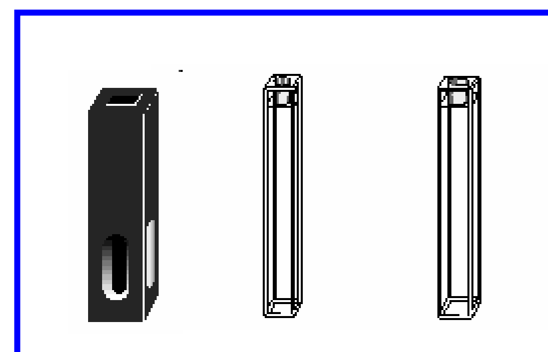






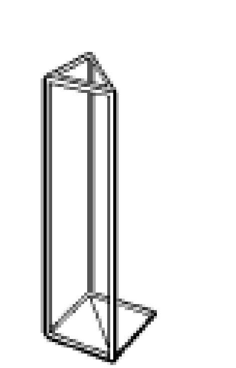
4 Position Turret
SPEX Fluoromax-2, Jobin-Yvon



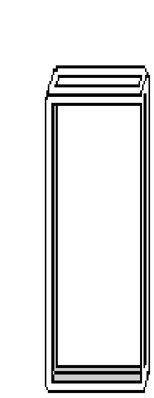


Front Face Detection

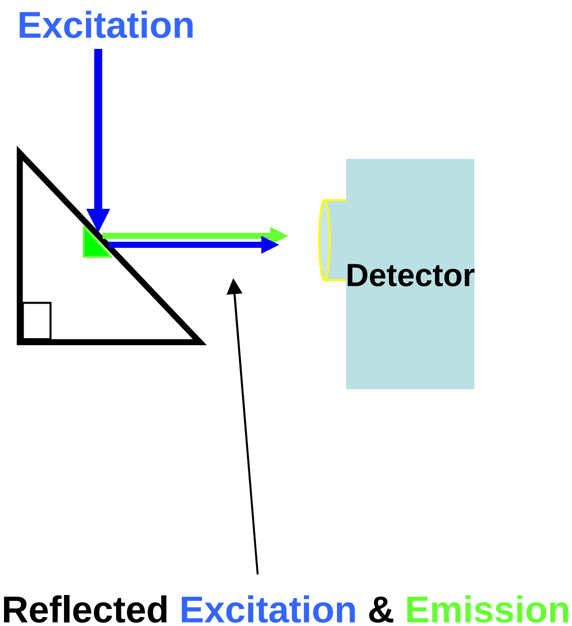
Triangular Cells

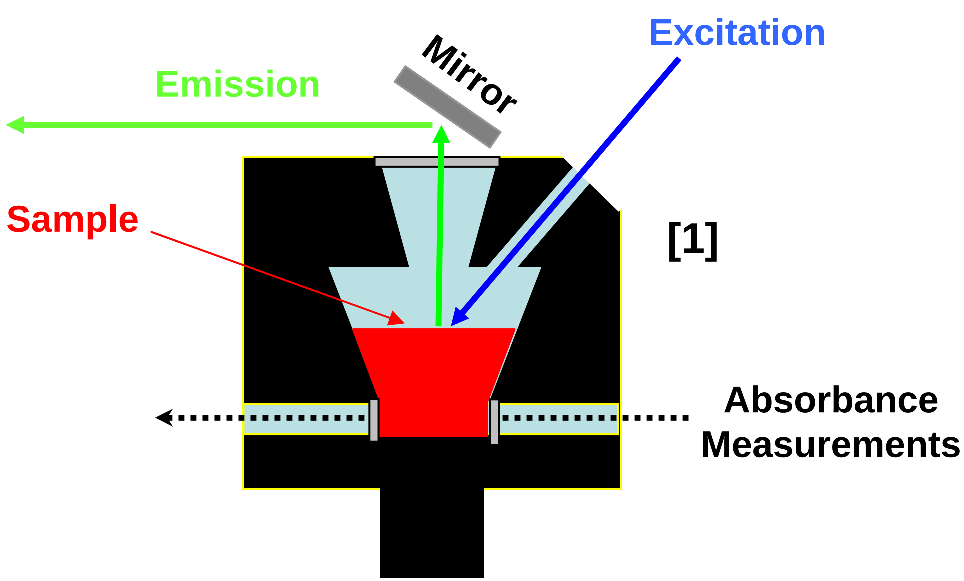


Thin Cells & Special Compartments



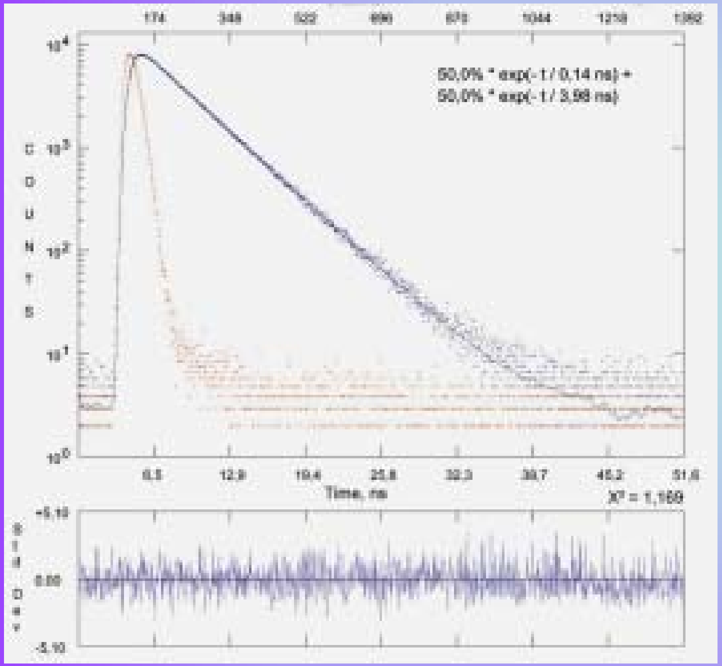
IBH, Glasgow G3 8JU
United Kingdom

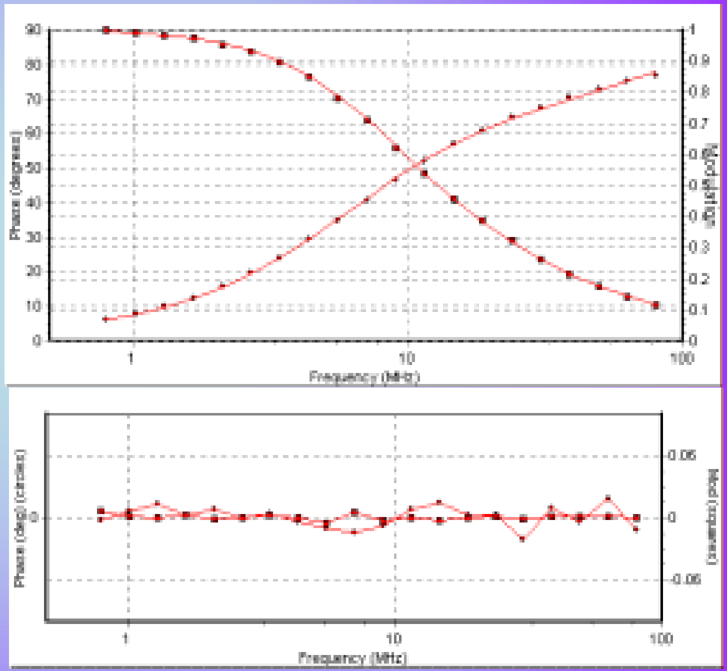




[1] Adapted from Gryczynski, Lubkowski, & Bucci Methods of Enz. 278: 538

Lifetime Instrumentation





Light Sources for Decay Acquisition: Frequency and Time Domain Measurements

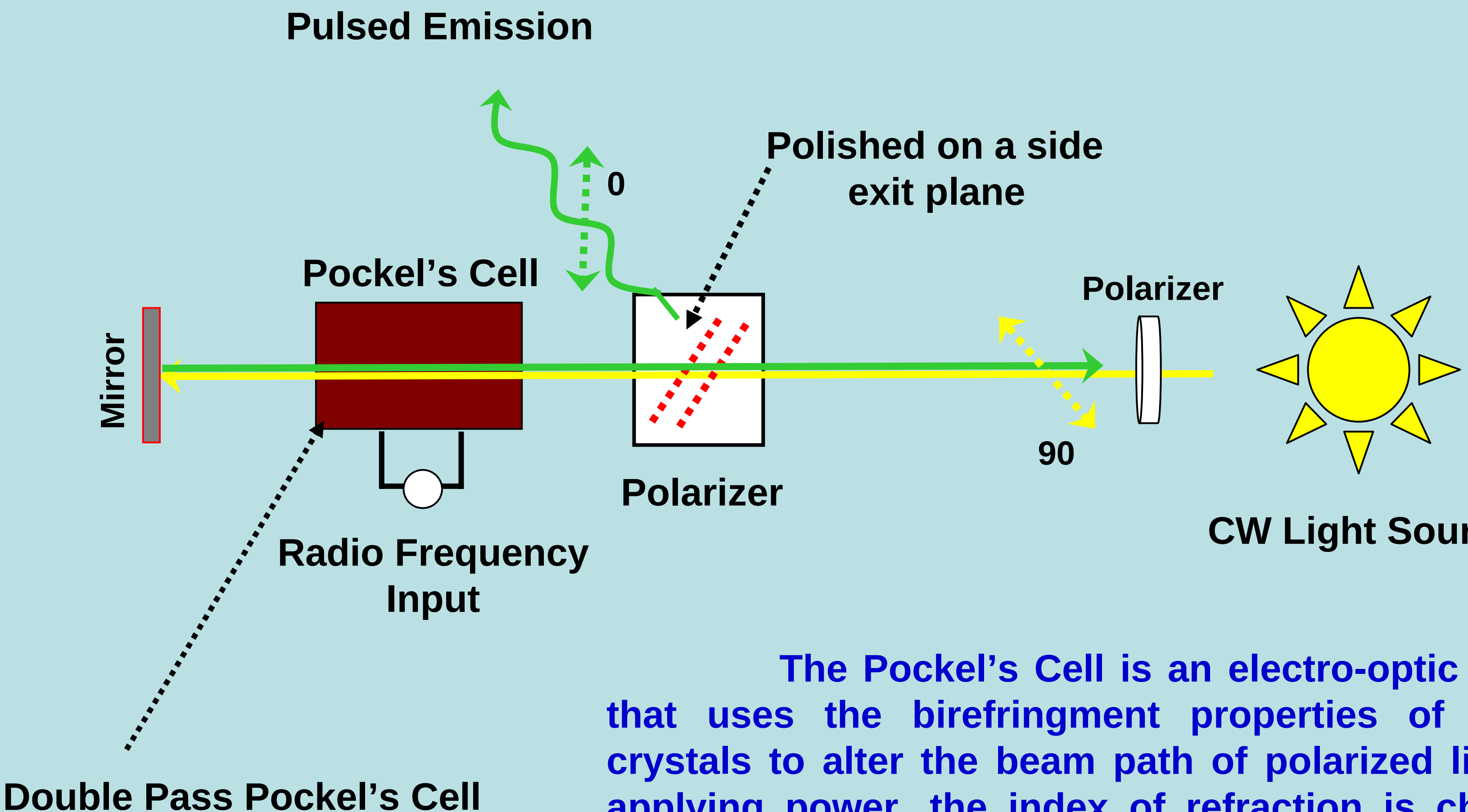
Pulsed Light Sources (frequency & pulse widths)

Mode-Locked Lasers
- ND:YAG (76 MHz) (150 ps)
- Pumped Dye Lasers (4 MHz Cavity Dumped, 10-15 ps)
- Ti:Sapphire lasers (80 MHz, 150 fs)
- Mode-locked Argon Ion lasers

Directly Modulated Light Sources
- Diode Lasers (short pulses in ps range, & can be modulated by synthesizer)
- LEDs (directly modulated via synthesizer, 1 ns, 20 MHz)

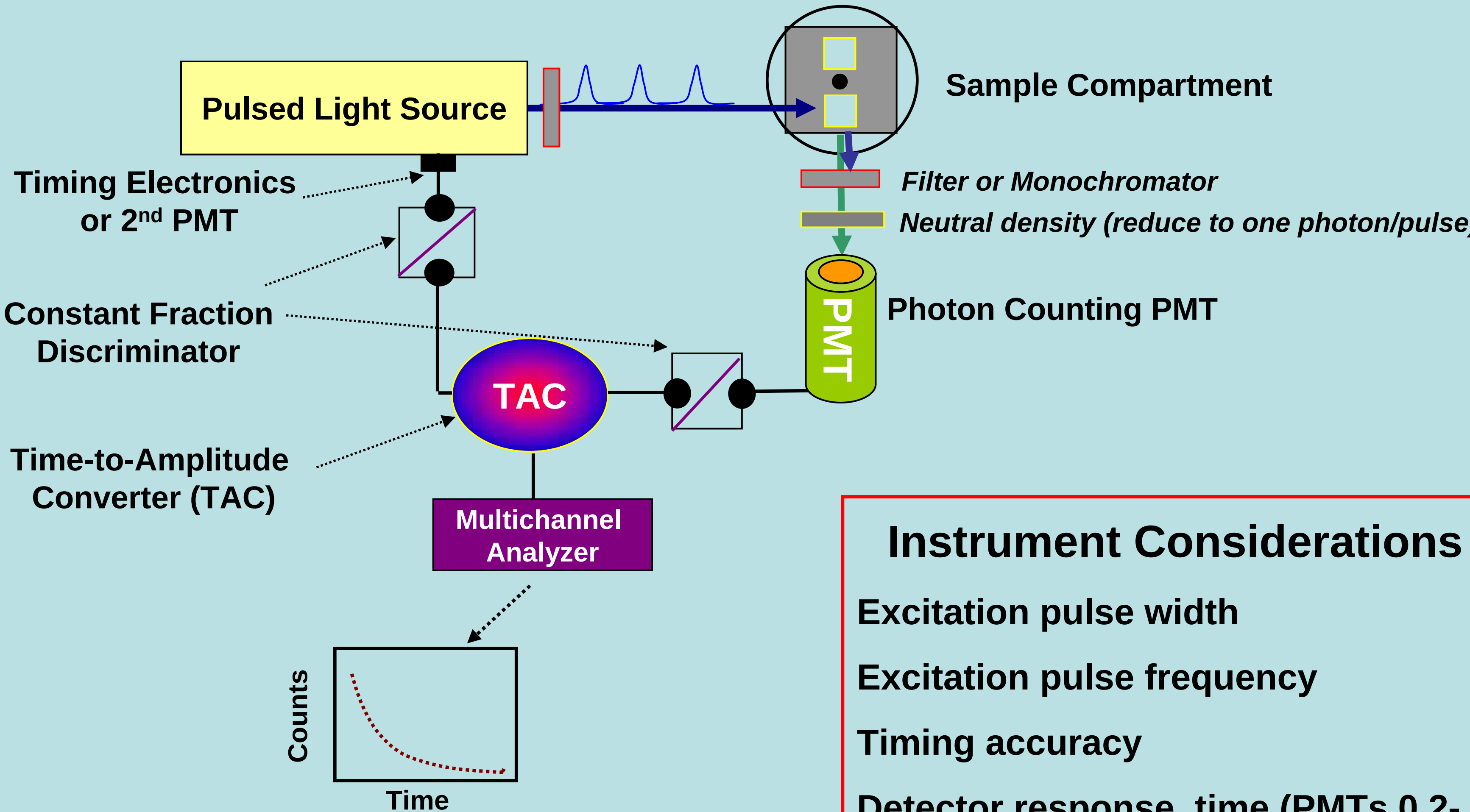
Flash Lamps
- Thyratron-gated nanosecond flash lamp (PTI), 25 KHz, 1.6 ns
- Coaxial nanosecond flashlamp (IBH), 10Hz-100kHz, 0.6 ns

Modulation of CW Light
Use of a Pockel's Cell



The Pockel's Cell is an electro-optic device that uses the birefringment properties of calcite crystals to alter the beam path of polarized light. In applying power, the index of refraction is changed and the beam exiting the side emission port (0 polarized) is enhanced or attenuated. In applying RF the output becomes modulated.

Time Correlated Single Photon Counting



Instrument Considerations

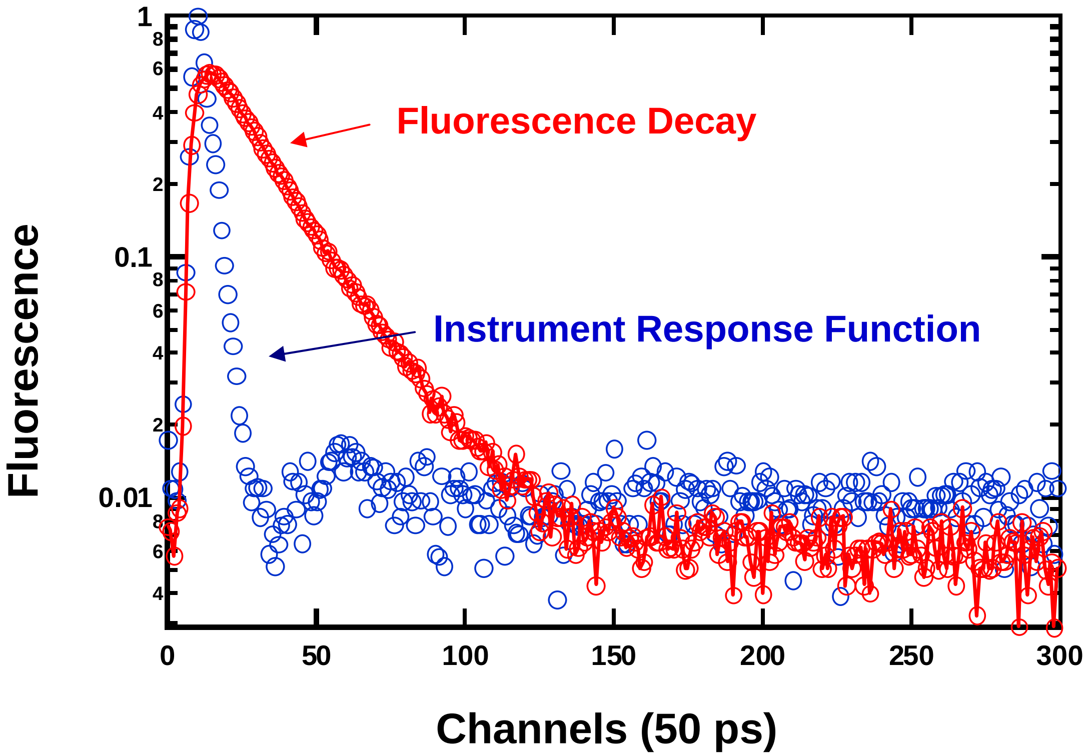
Excitation pulse width

Excitation pulse frequency

Timing accuracy

Detector response time (PMTs 0.2-0.9 ns; MCP 0.15 to 0.03 ns)

Histograms built one photon count at a time …



(1) The pulse width and instrument response times determine the time resolution.

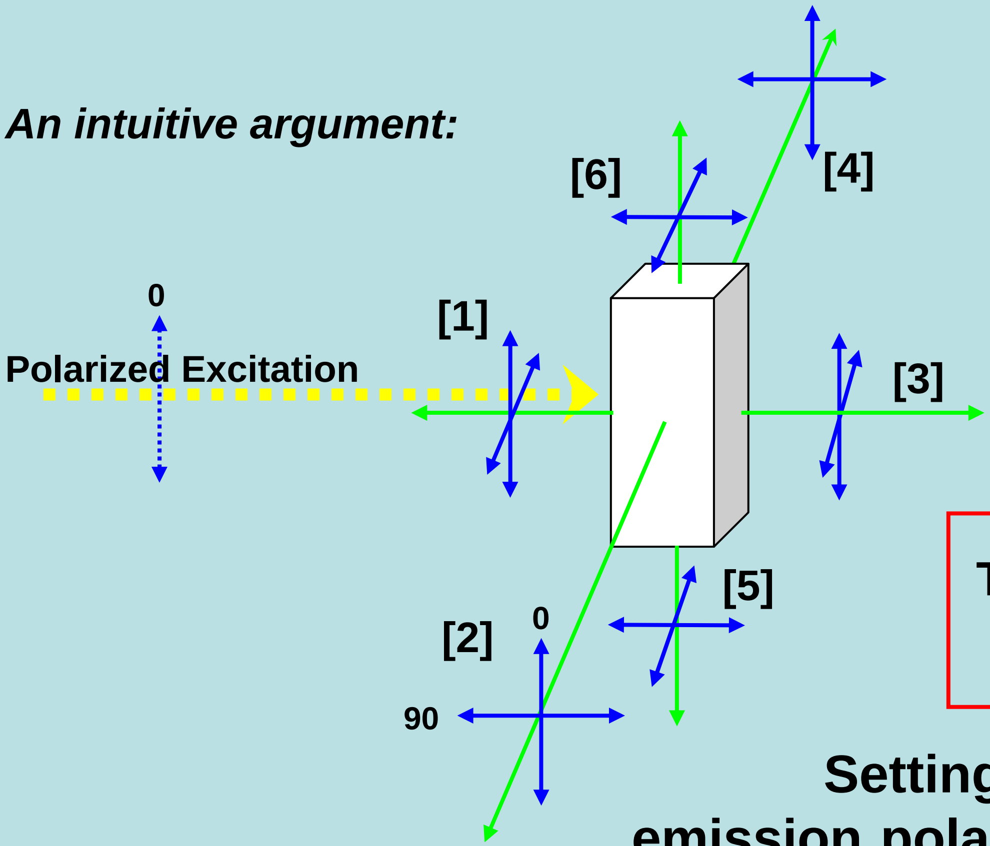
(2) The pulse frequency also influences the time window. An 80 MHz pulse frequency (Ti:Sapphire laser) would deliver a pulse every 12.5 ns and the pulses would interfere with photons arriving later than the 12.5 ns time.

Polarization Correction

There is still a polarization problem in the geometry of our excitation and collection (even without a monochromator)!!

Will the corrections never end ???

An intuitive argument:



$[1] = I_0 + I_{90}$
$[2] = I_0 + I_{90}$
$[3] = I_0 + I_{90}$
$[4] = I_0 + I_{90}$

$[5] = \quad 2 \times I_{90}$
$[6] = \quad 2 \times I_{90}$

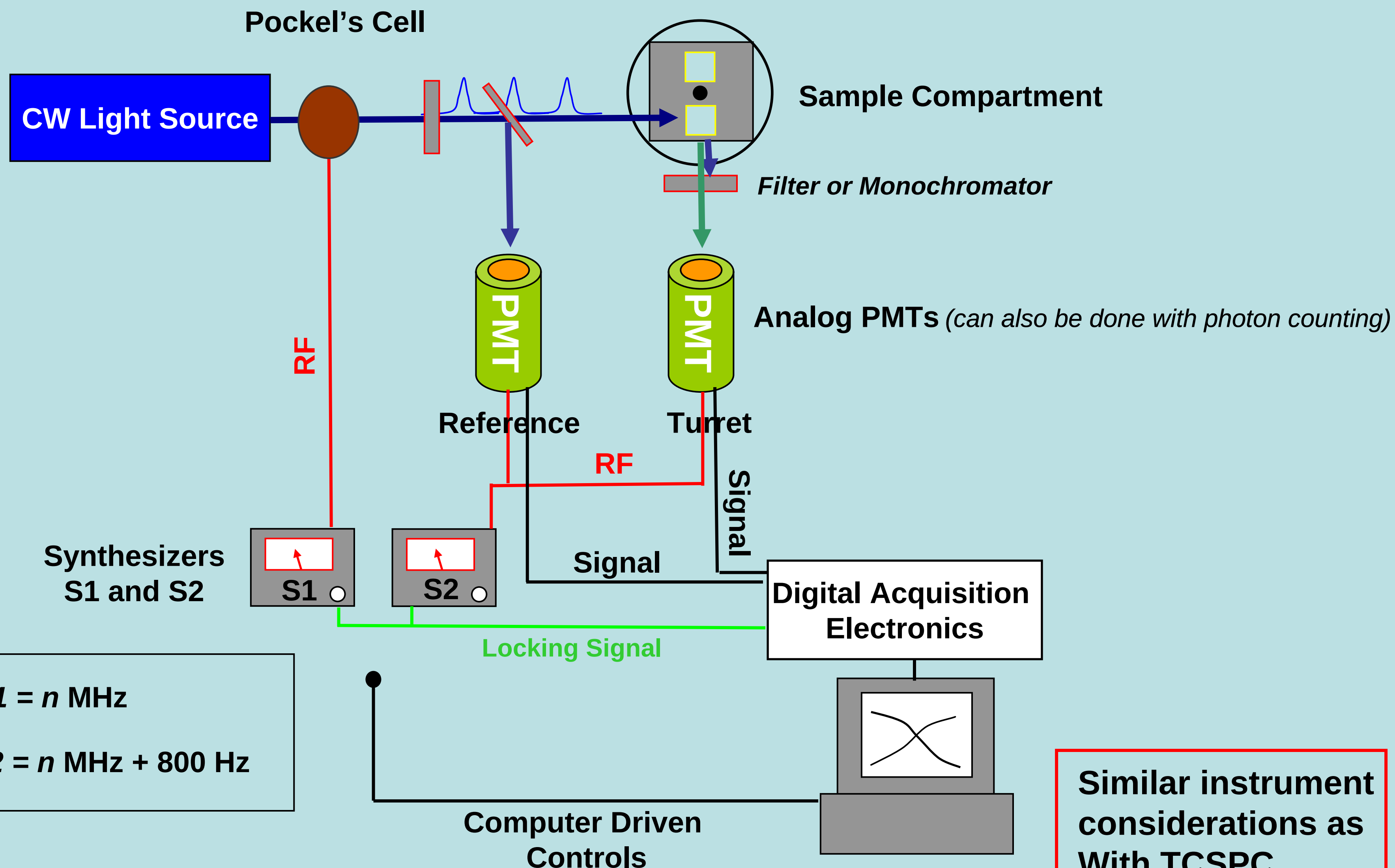
$\text{Total} = 4 \times I_0 + 8 \times I_{90}$

The total Intensity is proportional to:
$I_0 + 2 \times I_{90}$

Setting the <u>excitation angle to 0</u> and the <u>emission polarizer to 54.7</u> the proper weighting of the vectors is achieved.*

*Spencer & Weber (1970) J. Chem Phys. 52:1654

Frequency Domain Fluorometry



S1 = *n* MHz

S2 = *n* MHz + 800 Hz

Similar instrument considerations as With TCSPC

Lifetime Station #3, LDF, Champaign IL, USA

& hiding under the table: